EFFECTS OF CERTAIN ACID GROWTH-REGULATING SUBSTANCES AND THEIR CORRESPONDING ALDEHYDES ON THE GROWTH OF ROOTS[1]

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 620

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Introduction

In this study the effects on root elongation of two acid growth-regulating substances, 3-indoleacetic acid (IAAcid) and 1-naphthaleneacetic acid (NAAcid), were observed and compared with those of their corresponding aldehydes, 3-indole acetaldehyde (IAAld) and 1-naphthalene acetaldehyde (NAAld). Equivalent studies have been made earlier for the acids but not for the aldehydes. Intact roots, occasionally containing appreciable amounts of growth substances (**13, 33**), elongate less than normally when treated with any of several growth substances in concentrations greater than approximately 10 μgm/l (**3, 4, 16, 25**). At very low concentrations—0.001–1 μgm/l—stimulation of the growth of roots of intact seedlings has been reported (**7, 8, 22, 23, 25, 26, 32**). Initially, therefore, in the present investigation the relationships between concentration of growth substance and rate of root elongation were determined. Each compound was tested over a range of concentrations of which the lowest had no detectable effect on the roots, while the highest strongly inhibited their elongation.

The findings of an inhibition of root growth by IAAld and NAAld raised the question whether these aldehydes are active directly as auxins or must first be converted to acid auxins as indicated by the results of LARSEN (**18**) using *Avena* coleoptile material. The answer was sought through experiments in which given amounts of NAAld were incubated with root juice, and the mixture was analyzed for both neutral and acid growth substances after given periods. A decrease in neutral and an increase in acid growth-substance concentrations with time indicate conversion of the aldehyde to the acid by the root juice.

Relatively high concentrations of growth substances may cause subterminal swellings of root tips (**9, 20**). Such swellings were also observed in the present study in roots strongly retarded in linear growth, phenomena frequently accompanied by retarded root-hair formation. Preliminary microscopical studies were carried out on both intact and sectioned root material to clarify anatomical and cellular detail associated with these formative effects.

Material and methods

The present investigations aimed at establishing a micro-assay method for growth substances based on effects on root elongation. A number of small-seeded species were initially chosen as suitable for the test procedures described later: *Artemisia absinthium* L., *Lobelia*

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erinus var. *compacta* (Kaiser Wilhelm), *Mentha crispa* L., × *Mimulus tigrinus grandiflorus*, *Nicotiana* sp. (a Danish variety),[2] *Nicotiana sylvestris* Spegaz. and Comes, *Nicotiana tabacum* L. (Smyrna no. 23), *Nicotiana tabacum* L. (two varieties), *Papaver* sp. (Orientale Red), *Petunia* sp. (Howard's Star Improved), and *Sporobolus airoides* Torr. All species showed inhibition of root elongation by indoleacetic acid at 100 μgm/l.

Of these species, *Artemisia absinthium* was selected for the bulk of the investigations reported herein because of an acceptable germination percentage and rate of growth, an externally demarcated hypocotyl-root juncture, and rather straight growth of roots when the achenes are initially oriented with the radicle pointing downward. In planting, dry *Artemisia* achenes were oriented in rows on moist filter paper in a 9-cm. Petri dish so that the radicles would be pointing downward when the Petri dish was placed vertically in a holder. For the first 3 hours after moistening, the achenes were exposed to light of approximately 180 foot-candles from a 40-watt incandescent bulb with reflector 11 inches distant from the dishes. Temperatures within the dishes were not measured during these periods. The germination percentage (50–70%) of achenes so treated was two to three times that of nonilluminated achenes. After this initial illumination, the achenes and seedlings were kept either in darkness or in red light (Corning light filter no. 243) for handling and measurement. At the end of 48 hours in the vertical position, seedlings were selected for testing. Ten plants, the roots of which were straight and approximately 1 mm. long, were spaced equally, parallel one to another, upon a prepared agar square in each test, and the subsequent growth was observed during a period of 24 hours. Growth tests were carried out in a dark room with controlled temperature (22°–23° C.) and humidity (65% relative humidity) as a matter of convenience and precaution, as seedlings at controlled temperatures exposed to moderate white laboratory light showed the same relative growth responses in several, but not all, parallel experiments.

In preparing the agar, shreds of U.S.P. agar-agar were first washed continuously for 3 days in tap water and with several successive changes in glass-distilled water. After drying, known weights were made up to a given concentration with water, and the bulk was then autoclaved and subdivided. Agar plates 100 × 100 × 1 mm. in dimension were cast by pouring 10 ml. of melted agar upon a hot 10-cm.-square glass plate. The final composition of each agar plate was: 1.25% agar, 0.005 *M* Sörensen's citrate buffer (58.7 ml. of 0.1 *M* citrate plus 41.3 ml. of 0.1 *N* NaOH), and 0.001 *M* $CaCl_2$.

From such a plate, individual 1-cm. squares with a volume of 0.1 ml. were cut with a parallel-bladed cutter and placed upon numbered microscope slides. These squares, untreated, were used for control tests. Growth substances were added to other squares by the ether-dropping method described by Boysen Jensen (**6**) and Larsen (**17**). The ethyl ether used was Mallinckrodt no. 0848, Ether Anhydrous Analytical Reagent, periodically redistilled to remove peroxides prior to use in the laboratory.

The growth substances were dissolved in such ether, and appropriate dilutions were made. A measured quantity of a given solution was evaporated at 55°–60° C. in a test tube through which air

[2] Seed of the first five species listed was furnished through the courtesy of J. E. Ohlsen's Enke, Seed Growers, Copenhagen, Denmark.

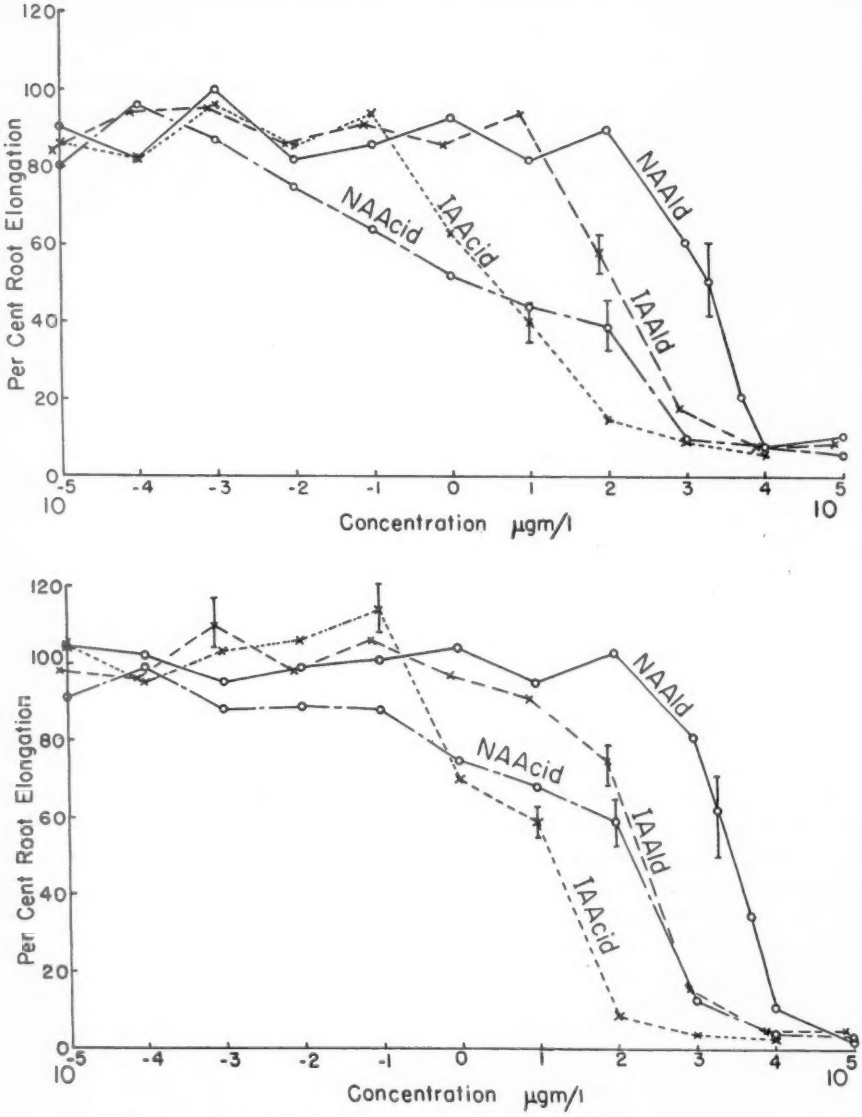
previously dried with $CaCl_2$ was passed; the residue was immediately dissolved in 0.5 ml. of ether. This new solution was drawn up into a pipette and dropped slowly upon the agar square, which was placed over a water bath at 45°–50° C. A stream of dry air, which passed through a jacket surrounding the ether pipette, hastened the evaporation of the ether as it was dropped on the square. An additional 0.3 ml. of ether, used to rinse the solution tube and pipette, was dropped on the same square. When all the ether had been dropped, the slides with agar squares were placed in a saturated atmosphere in Petri dishes lined with moist filter paper to stand for 1 or more hours. During this time uniform distribution of the growth substance within each square was attained. All preparation of the agar up to this point was carried out in the laboratory in white light.

Of the growth substances used, IAAcid was a commercial preparation of Eastman Kodak, NAAcid of Merck and Company. The NAAld was part of a preparation furnished by Professor K. A. JENSEN, University of Copenhagen, Denmark (**14, 15**), made according to method 3 of JENSEN and CHRISTENSEN (**14**:704). The IAAld was prepared according to the methods outlined by LARSEN (**17**: 85). The procedure followed in preparing the IAAld sample was to dissolve 8 mg. of isatin in 2 ml. of water, for which moderate heating was necessary. This solution was transferred to a flask containing 7.3 mg. of L-tryptophane and 1 gm. of KH_2PO_4 dissolved in 10 ml. of water. The resulting mixture was refluxed at 100° C. for 30 minutes, cooled, and to it was added 1 ml. of 15% tartaric acid. It was then shaken out three times, 4 minutes each time, with successive 15-ml. portions of ether.

In order to remove all acid substances, the ether solution, after being reduced in volume to 25 ml., was shaken out three times with alkaline water (20 ml. of water plus 1 ml. of 8% $NaHCO_3$, each time). The remaining ether was then made up to volume in a 25-ml. volumetric flask. The yield was computed as ten times the number of μgm.-"equivalents" in the sample, based on determinations in the *Avena* test at 15° curvature (**17, 18, 19**). In this preparation, a 0.97% yield was obtained. The L-tryptophane and isatin used were commercial preparations of Eastman Kodak Company.

For measuring elongation of the seedlings on the agar squares an initial root length was recorded using an ocular micrometer at $\times 5\frac{1}{4}$. Further measurements were made 4 and 24 hours later. Between measurements the slides were placed vertically in Coplin staining jars, all roots pointing straight downward. A nearly saturated atmosphere was maintained in the jars by lining the inner surface of the covers with wet filter paper and by placing about a centimeter of water in the bottom.

The data for individual root lengths at 0, 4, and 24 hours were used to compute the increases in length over each time period. The elongation of treated roots was expressed as a percentage of that of the untreated roots (table 2; figs. 1, 2). (Untreated roots grew approximately 0.14 mm. per hour for the first 4 hours, 0.19 mm. per hour for the subsequent 20 hours, and 0.18 mm. per hour for the 24 hours.) Vertical bars in figures 1 and 2 are erected at those concentration levels giving approximately 50% growth to show the 95% confidence intervals of the percentage elongation values for those concentrations. The intervals chosen delimit the growth values between which and the given mean



FIGS. 1, 2.—Effects of several concentrations of aldehydes and acid growth substances on elongation of seedling roots of *Artemisia absinthium* at 22° C. expressed as percentage of elongation of untreated roots. Vertical bars represent 95% confidence intervals. Fig. 1 (*above*), growth for 4 hours. Fig. 2 (*below*), growth for 24 hours.

value no significant difference would be found at the 5% level in a t test. The confidence intervals for the ratios (percentages) were computed from formulas (**2, 29**) based on the method given by FISHER (**11**:§ 62.1, pp. 192–193).

Experiments testing conversion of NAAld to NAAcid by root juice were based on techniques of LARSEN (**18, 19**). The procedure was to grind up roots of 4-day-old seedlings in a mortar containing 0.01 M primary phosphate buffer solution, centrifuge the resulting mixture for 20 minutes at 0° C., filter the supernatant through a coarse, fritted Pyrex glass filter, and incubate aliquots of the juice filtrate with solutions of growth substance in 0.01 M phosphate buffer for given time periods. Neutral and acid fractions of growth substance were then recovered from the juice–growth substance mixture (pH 5.8) by shaking out with ether, first at an alkaline (pH 8.4–9.0) and then at an acid (pH 1.2–2.2) reaction. In these shakings the bulk of the growth substances is recovered in the ether. The ether fractions were made up to 25-ml. volume and tested with the *Avena* coleoptile curvature method. Amounts of neutral and acid growth substance in the fractions were determined using standard curves in which degrees curvature in the *Avena* test were plotted against concentration of NAAld and NAAcid, respectively. Recovery of growth substance from aqueous solutions was tested at more than one concentration, and only curvatures smaller than the maximum angle are used for calculating amounts recovered.

An example of the procedure followed in the calculation of growth-substance recovery from conversion experiments is given (table 1), using data from *Avena* tests of May 27, 1950. The same incubation procedure was also used to determine the stability of NAAcid in solutions of root juice (table 5) and to demonstrate the inactivity of boiled juice.

TABLE 1

DETERMINATIONS OF AMOUNTS OF GROWTH SUBSTANCE RECOVERED FROM ARTEMISIA ROOT JUICE*

Reaction mixture	Incubation time (hr.)	Degrees curvature in Avena test	Equivalent growth substance conc. $M \times 10^{-9}$/ml of agar (from standard curves)	Milliliters tested	Recovery in $M \times 10^{-9}$†
Neutral fraction:					
0.01 M KH_2PO_4	2	23.6	20.81	5	10.40
Root juice	2	18.0	16.93	10	4.24
Root juice	4‡				
Acid fraction:					
0.01 M KH_2PO_4	2	13.2	0.731	2	0.914
Root juice	2	10.9	0.672	1	1.68
Root juice	4	13.3	0.737	0.8	2.30

* 14.7×10^{-9} M of NAAld dissolved in 1 ml. of phosphate buffer solution was mixed with 1 ml. of buffer solution or 1 ml. of diluted root juice and incubated in open test tubes at 22° C.

† Recovery $= \dfrac{M \times 10^{-9}\text{/ml. of agar} \times 0.1 \text{ ml. of agar} \times 25 \text{ ml. total volume of fraction}}{\text{ml. tested}}$.

‡ This solution was earlier exhausted, 17 ml. showing less than 0.94×10^{-9} M NAAld.

Results

Elongation of roots

All four growth substances were found to bring about lessened *Artemisia* root growth in certain concentrations (table 2; figs. 1, 2). A possible stimulatory effect at very low concentrations has been statistically established for IAAcid and IAAld. The aldehydes were found generally to be one-tenth as effective in moderately inhibiting root growth as were acid growth substances. NAAld had the lowest activity in this respect of the four compounds, being approximately one-tenth as effective as IAAld. NAAcid, on the other hand, induced varying responses with respect to the other compounds over its range of effective concentrations. The chief difference was that it exhibited an inhibitory effect upon root growth even at concentrations of 10^{-2} μgm/l. All four substances were strongly inhibitory at high concentrations (figs. 1, 2).

Root growth was less inhibited by contact with a given concentration of growth substance over the 24-hour than over the 4-hour period. Experiments on persistence of growth substance in the agar showed that 10^{-3} μgm. of IAAcid virtually disappeared from an agar square upon which roots had grown for 24 hours. Other than a lessened sensitivity to a given concentration, root responses to inhibiting concentrations were rather similar in the 24-hour period to those of the 4-hour period. A major difference was seen in the fact that only over 24-hour periods did roots show a stimulation, apparent or real, of growth. An apparent explanation, namely, that the 24-hour stimulation was due to an exhaustion of the growth substance in the agar to a stimulatory level seems implausible from the fact that successively

TABLE 2

ROOT GROWTH (PERCENTAGE OF CONTROL) OVER 4 AND 24 HOURS AS AFFECTED BY GROWTH SUBSTANCES

Growth substance conc. (μgm/l)	4 hours								24 hours							
	IAAld*		IAAcid		NAAld		NAAcid		IAAld*		IAAcid		NAAld		NAAcid	
	K†	%	K†	%	K†	%	K†	%	K†	%	K†	%	K†	%	K†	%
10^{-6}	.....	.....	(3)	86	(2)	79	(3)	87	.....	.....	(3)	103	(1)	94	(3)	102
10^{-5}	(2)	84	(2)	86	(2)	90	(2)	80	(2)	98	(2)	104	(2)	104	(2)	91
10^{-4}	(4)	94	(3)	82	(3)	82	(6)	96	(4)	96	(3)	95	(2)	102	(6)	99
10^{-3}	(4)	95	(2)	96	(2)	100	(3)	87	(4)	110	(2)	103	(2)	95	(3)	88
10^{-2}	(4)	86	(6)	86	(3)	82	(2)	75	(4)	99	(6)	106	(2)	99	(2)	89
10^{-1}	(6)	91	(5)	94	(3)	86	(3)	64	(6)	106	(5)	114	(3)	101	(3)	88
0	(4)	86	(2)	63	(2)	93	(2)	52	(4)	97	(2)	70	(2)	104	(2)	75
10	(3)	94	(7)	40	(3)	82	(2)	44	(3)	91	(7)	59	(3)	95	(2)	68
10^{2}	(3)	58	(2)	15	(3)	90	(3)	39	(4)	75	(2)	9	(3)	103	(3)	59
10^{3}	(5)	18	(3)	9	(3)	61	(3)	10	(6)	16	(3)	4	(3)	81	(3)	13
$10^{3\cdot3}$	.....	.....	.....	.....	(2)	51	.....	.....	.....	.....	.....	.....	(2)	62	.....	.....
$10^{3\cdot7}$	.....	.....	.....	.....	(2)	21	.....	.....	.....	.....	.....	.....	(2)	35	.....	.....
10^{4}	(3)	8	(2)	6	(3)	8	(2)	8	(3)	5	(2)	3	(3)	11	(2)	4
10^{5}	(2)	9	.....	.....	(2)	11	(2)	6	(2)	5	.....	.....	(2)	2	(2)	3

* Subtract $10^{0\cdot2}$ from all conc. for IAAld. This correction has been made on the curves in figures 1 and 2.

† K equals number of agar squares tested, 10 (9, 8) roots per square.

lower concentrations still failed to give stimulation over a 4-hour period. Agar squares dropped with pure ether gave no stimulation. Stimulation of elongation observed at 24 hours has been statistically established at the 1% level for 10^{-1} μgm/l IAAcid (114% elongation, confidence interval 106–124%), and for $10^{-3.2}$ μgm/l IAAld (110% elongation, confidence interval 102–119%). IAAld, however, did not give stimulation at the next higher and next lower concentrations tested.

TABLE 3

RELATIVE GROWTH RESPONSES OF ARTEMISIA ROOTS AND AVENA COLEOPTILES

Growth substance	Effective concentration of growth substance (μgm/l)		
	Artemisia root test 50% elongation		Avena coleoptile test 15° curvature
	4 hours	24 hours	2.5 hours
IAAld	$10^{2.1}$	$10^{2.3}$	$10^{2.3}$
IAAcid	$10^{0.6}$	$10^{1.2}$	$10^{1.3}$
NAAld	$10^{3.3}$	$10^{3.5}$	$10^{3.4}$
NAAcid	$10^{0.2}$	$10^{2.2}$	$10^{2.2}$

All four substances were also tested in the inhibitory range of concentrations with *Nicotiana tabacum* as a test plant. Fifty per cent root growth was attained with concentrations of the four substances ranging from 10 to 10^3 μgm/l. Owing to difficulties in handling and to the greater variability of this species under the given conditions as compared with *Artemisia*, no further studies have been carried out with tobacco.

A quantitative comparison of growth-substance effectiveness in both the *Artemisia* root-growth test and the *Avena* coleoptile-curvature test is given in table 3. For the 24-hour *Artemisia* readings at the levels of comparison chosen, there were striking similarities of response to all four substances by the two organs concerned. The computations of the IAAld concentrations in both instances and throughout the paper are based on the assumption that 1 μgm.-"equivalent" of IAAld is equal to 10 μgm. of IAAld on a weight basis, a ratio derived from conversion experiments using NAAld (**18, 19**). A modification of this relationship would affect conclusions drawn from the root-growth tests relatively little, as these results are based on the logarithmic expression of concentrations of growth substances.

CONVERSION OF ALDEHYDES TO ACIDS

The presence of a system in the root juice capable of converting aldehyde growth substances to acids under the given experimental conditions was demonstrated. Synthetic NAAld was used because the results could then be expressed directly in absolute weight units, while amounts of IAAld may only under certain assumptions (**19**) be so expressed. There occurred during the time of incubation of NAAld with the juice a progressive increase in amount of acid growth substance and a concurrent decrease in amount of aldehyde in the mixture (table 4). The total amount of growth substance present in the mixture decreased. That this decrease was not occasioned by a destruction of the acid growth substance formed is shown in table 5. Preliminary experiments indicated no conversion of NAAld to acid growth substance by boiled root juice.

LARSEN (**19**) has shown that two moles of NAAld yield one mole of NAAcid when treated with coleoptile juice. This reaction is probably a dismutation, leading to the formation of one mole of

acid and one mole of alcohol. If the conversion process in the root juice were of the same nature, one would expect, after the aldehyde had completely disappeared, a final yield of acid equaling one-half of the original amount of aldehyde when expressed on a molar basis. (One μgm. of NAAld equals 5.9×10^{-9} *M*; 1 μgm. of NAAcid, 5.4×10^{-9} *M*.) The yield of acid, however, after the amount of aldehyde had become undetectably low, was considerably less than 50%.

TABLE 4

CONVERSION OF NAPHTHALENE ACETALDEHYDE TO NAPHTHALENEACETIC ACID IN ROOT JUICE*

Reaction mixture	Incubation time (hr.)	Growth substance recovery $M \times 10^{-9}$			Percentage recovery
		Neutral fraction†	Acid fraction†	Total	
Experiment 1:‡					
0.01 *M* KH_2PO_4	2	13.00	0.38	13.38	91
Root juice	1	9.06	0.48	9.54	65
Root juice	3	6.83	1.08	7.91	54
Experiment 2:§					
0.01 *M* KH_2PO_4	2	10.48	0.97	11.45	78
Root juice	2	4.12	1.72	5.84	40
Root juice	4‖	$\leqq$ 0.94	2.26	$\leqq$ 3.20	$\leqq$22

* 14.7×10^{-9} *M* of NAAld dissolved in 1 ml. of phosphate buffer solution was mixed with 1 ml. of buffer solution or 1 ml. of diluted root juice and incubated in open test tubes at 22° C.

† Each figure based on a minimum of two *Avena* tests.

‡ Juice from 0.078 gm. of roots diluted with 3 ml. of 0.01 *M* KH_2PO_4 solution.

§ Juice from 0.445 gm. of roots diluted with 3 ml. of 0.01 *M* KH_2PO_4 solution.

‖ No curvature obtained with two-thirds of the fraction. Threshold value for NAAld is given.

TABLE 5

STABILITY OF NAPHTHALENEACETIC ACID IN ROOT JUICE*

Reaction mixture	Incubation time (hr.)	Percentage recovery
Experiment 1:†		
0.01 *M* KH_2PO_4	0	76
Root juice	0	80
Root juice	4	84
Experiment 2:‡		
0.01 *M* KH_2PO_4	0	74
Root juice	0	80
Root juice	4	104

* 2.7×10^{-9} *M* of NAAcid dissolved in 1 ml. of phosphate buffer solution was mixed with 1 ml. of buffer solution or 1 ml. of diluted root juice and incubated in open test tubes at 22° C.

† Juice from 0.526 gm. of roots diluted with 2.5 ml. of 0.01 *M* KH_2PO_4 solution.

‡ Juice from 0.570 gm. of roots diluted with 3.85 ml. of 0.01 *M* KH_2PO_4 solution.

Hence, it may be concluded that NAAld was also changed in some other way than by conversion to acid. The fact that NAAld was ineffective in inhibiting root elongation at concentrations lower than 10^2 μgm/l while an effect of NAAcid could be observed at 10^{-2} μgm/l—a difference of 10^4 μgm/l—indicated that very little "active" NAAcid was formed by roots either 4 or more hours after treatment with NAAld. Percentage inhibition by NAAld and IAAld was relatively uniform throughout the 24-hour period.

FORMATIVE EFFECTS

Nearly isodiametric swellings, approximately 0.26 mm. in diameter and

an equal distance back from the root tip, were observed on roots which had been in contact for 24 hours with relatively high concentrations of each of the growth substances—10^4 μgm/l for the acids, 10^5 μgm/l for the aldehydes (table 6). Such roots tended to resume a normal rate of growth over the following 24 hours when the agar squares were brought in contact with tap water. Root hairs, if formed at all, were irregularly distributed on the new growth under water.

In the only test using 10^6 μgm/l of growth substance (NAAld), no swellings appeared, the root tips turned brown, and the hypocotyls were badly stunted. Such stunting of hypocotyl growth was not observed with the usual range of concentrations of growth substances which brought about limited root growth. Seedlings, untreated or treated with growth substances, rarely produced lateral roots during a 3-week period of observations.

Production of root hairs was adversely affected by concentrations of growth substances which brought about swellings of the roots (table 6). Few root hairs were found on such roots. In inhibiting the production of root hairs, as in the effects on root elongation, the aldehydes were one-tenth as effective as the acids. In somewhat lower concentrations, those which brought about 10% root growth, the roots were characterized by the occurrence of a thick fuzz of root hairs. This apparent increase in the number of root hairs might have been due to the typical shortness of cells in such roots, in that there were more epidermal cells, and therefore more root hairs, per unit length of root. In sectioned material of roots treated with 10^3 μgm/l IAAld the epidermal cells were approximately one-third to one-fourth as long as in equivalent regions of untreated roots. Those treated roots whose average elongation was less than 20% that of those untreated had a thick fuzz of root hairs. No effects of the growth substance upon cell divisions, lateral or transverse, were noticed. In longitudinal sections of both untreated and treated material, more than ten consecutive epidermal cells were observed with root hairs, produced at the distal end of the cells, indicating no regular alternation of trichoblasts and atrichoblasts (30). In fact, atrichoblasts were difficult to find.

TABLE 6

FORMATIVE EFFECTS OF GROWTH SUBSTANCES
(24 hours after treatment)

GROWTH SUBSTANCE	EFFECTIVE CONCENTRATIONS (μGM/L)			
	Root hairs per unit length of root		Swellings	10% growth
	Abundant	Sparse		
IAAld	$10^{3.4}$	10^5	10^5	10^3
IAAcid	$10^{1.2}$	10^4	10^4	10^2
NAAld	$10^{3.4}$	$10^{5.6}$	10^5	10^4
NAAcid	10^3	$10^{4.5}$	10^4	10^3

In the limited experiments with *N. tabacum* relatively high concentrations of certain growth substances also brought about swellings and decreased root-hair production by seedling roots.

Discussion

The advantages of the *Artemisia* root-growth test may be considered: the growth of ten intact seedling roots can be measured on an agar square whose volume is 0.1 ml.; these roots are sensitive to growth substances over a wider range of concentrations than are *Avena* coleoptiles; this results in an applicable test for minute quantities of growth substance such as often result from plant extracts. Use of ether solutions makes

the reduction of volumes easy. The proportionality of response (inhibition of elongation) to the logarithm of concentration of growth substance is an advantage when testing over a wide range of concentrations is desired but a disadvantage if small differences in concentration of growth substance are to be detected. Daily variations in rate of growth render the use of a control necessary. Inherent variability of the test and the labor involved in carrying out a test are roughly equivalent to that of the *Avena* coleoptile test. First readings are taken at 4 hours, final readings at 24 hours.

The observed inhibition of *Artemisia* root growth by growth substances agrees with numerous earlier published reports on other species (**4, 16, 25, 27**). Unidentified plant auxins, IAAcid, NAAcid, and other substances have been so studied. A point of difference among these workers is the question of stimulation of root growth by growth substances, which has been reported by certain workers using cultures of excised roots (**4, 10, 12**), decapitated roots (**1**), and intact roots (**7, 8, 22, 23, 25, 26, 32**). MOEWUS (**25**), using a highly standardized auxin assay method with cress (*Lepidium sativum* L.) seedlings, reported statistically highly significant stimulation of root growth by IAAcid concentrations of from 10^{-3} to 10^{-1} μgm/l. NAUNDORF (**26**), growing seedlings of *Helianthus annuus* L. in aqueous solutions of IAAcid for 5-hour periods, reported a stimulation of root elongation at concentrations of 10^{-4} to 10^{-1} μgm/l, reaching nearly 100% at 10^{-3} μgm/l. In the present study *Artemisia* root growth was stimulated by concentrations of IAAcid from 10^{-3} to 10^{-1} μgm/l, and statistical significance has been established at the 1% level for stimulation obtained with 10^{-1} μgm/l. Stimulation of root growth has also been obtained using non-auxins such as HCl (**16**), Nekal BX (**28**), and $CuSO_4$ (**10**), which raises the question of whether the auxin activity in a root can be influenced by external factors, including growth substances. This question was not investigated in the present study.

The stimulation of root growth by growth substances fits in with the suggestions of BOYSEN JENSEN (**5**) and THIMANN (**31**) that roots differ from stems in having much lower optima for the effects of growth substances upon elongation. Reported instances of stimulation of the growth of intact roots indicate that some roots contain lower than optimal concentrations of auxin. That stimulation was observed only after 24 hours, but not after 4 hours, of exposure to low concentrations of growth substances may be explained on the basis of results obtained by BURSTRÖM (**7, 8**). He distinguished two phases of growth in roots of wheat, the first characterized by increasing elasticity and increasing turgor tension of the cell wall, the second by decreasing elasticity and a constant turgor tension. All effective concentrations of auxin (here IAAcid) stimulated the first phase of growth. Low concentrations, while stimulating the first phase, might be without effect on the second one, which, however, was inhibited by higher concentrations. Overall stimulation of root growth by low concentrations of auxin is explained as a result of stimulation of the first phase of growth and lack of inhibition of the second phase. Very little actual elongation takes place during the first phase, and this preparatory stage is completed sooner the higher the concentration of auxin. Assuming that the effect of auxin in stimulating the growth of roots of *Artemsia* is caused by an action on the first phase of growth, it is understand-

able, in view of BURSTRÖM's ideas, that stimulation occurs several hours after the application of auxin. Inhibition, on the other hand, which seems to be an effect directly on the rapidly elongating cells, already in their second phase of growth, is accomplished much sooner.

Four different growth substances were used in the present study. The particular closeness of agreement between their relative activities in the *Avena* coleoptile test, on the one hand, and in the *Artemisia* root test, on the other hand (table 3), indicated a rather fundamental effect of growth substances upon cell elongation. All four stimulated the elongation of cells in the *Avena* coleoptile; they inhibited the elongation of cells in *Artemisia* roots. Additional effects upon cell divisions were not observed. In neither test was a time lag in the response to aldehydes established, indicating that, if aldehydes must first be converted to acids to be effective as auxins, the conversion was rapid in living tissues. A difference between the two organs was that the response, which was directly linear to the concentration of growth substance in the *Avena* coleoptile test, was linear to the logarithm of the concentration of growth substance in the *Artemisia* root test.

Experiments testing conversion of aldehydes to acids showed, as in similar experiments with *Avena* coleoptile juice (**19**), a progressive decrease in the amount of NAAld which was incubated with root juice and a corresponding increase in the amount of acid recovered from such juice. Total recovery of growth substance was considerably less than would be expected, even assuming a dismutation of the aldehyde to form one mole of acid and one mole of alcohol (the latter undetectable in the *Avena* test) per two moles of aldehyde which disappear. In view of the stability of NAAcid in root juice these results suggest that root juice was able to change NAAld in some manner other than by an oxidation or a dismutation of the molecule yielding acid auxin. The actual processes involved in the living root may differ both qualitatively and quantitatively from those demonstrated in root juice.

Subterminal root swellings induced by growth substances in relatively high concentrations have often been reported in the auxin literature (**9, 20**)—a response brought about by decreased longitudinal and increased radial extension of cells. LEVAN and LOTFY (**21**) found in *Allium* that the region which formed the swellings in treated roots became the zone of elongation in untreated roots. These authors postulated that growth substances are chiefly effective upon cells undergoing differentiation, meristematic cells being more resistant to the effects of auxin.

In contrast to the marked effect of growth substances upon longitudinal extension of cell walls, formation of root hairs appeared to be affected relatively little except when high concentrations of these substances were employed. This refers to the presence or absence of root hairs, not to the length of given root hairs. On the question of the effect of growth substances upon individual root hairs, MEESTERS (**24**) reported root hairs to be inhibited about 20% by concentrations of growth substances which gave maximum root inhibition. In the present study production of root hairs became negligible at those concentrations at which longitudinal growth of roots had practically ceased and swellings were present. With lesser concentrations, at which root growth was strongly inhibited, the length of individual epi-

dermal cells was shorter than normal, and root hairs were still present. Preliminary microscopical observations suggested that the ratio of the number of root hairs to that of epidermal cells was not affected by these concentrations of growth substances. Thus, treated roots, containing more epidermal cells and therefore more root hairs per millimeter of root length, had a thick fuzz of root hairs when compared with untreated roots. With all four substances the concentrations correlated with the root-hair fuzz were those allowing 10% growth.

The ability of rather severely inhibited roots to return to apparently normal growth is consistent with the postulate of LEVAN and LOTFY (21) that root meristematic cells are resistant to the effects of auxin and can endure such effects until the return of favorable growing conditions, at which time normal growth of the roots will be resumed. Portions of the roots which formed swellings did not recover a normal thickness; this supports the hypothesis that the new root growth is the result of renewed meristematic activity.

Summary

1. Differential elongation of seedling roots of *Artemisia absinthium* in response to applied growth substances was used as the basis of a method for assay of growth substances. The use of only 0.1-ml. agar squares for testing the growth of ten roots and marked sensitivity of the roots render the method applicable for small quantities of growth substances.

2. The elongation of roots treated with four substances—3-indoleacetic acid (IAAcid), 1-naphthaleneacetic acid (NAAcid), 3-indole acetaldehyde (IAAld), and 1-naphthalene acetaldehyde (NAAld)—was expressed as a percentage of the elongation of untreated roots.

3. All four substances showed an inhibitory effect on root elongation over a certain range of concentrations (figs. 1, 2). In general, the aldehydes were one-tenth as effective in inhibiting root elongation as their corresponding acids. 1-Naphthalene acetaldehyde was one-tenth as effective as 3-indole acetaldehyde. 1-Naphthaleneacetic acid was less effective than 3-indoleacetic acid at highly inhibitory concentrations, more effective at slightly inhibitory concentrations.

4. For all four compounds, given concentrations were more inhibitory at 4 than at 24 hours. In no instance did elongation of treated roots exceed that of those untreated at the end of a 4-hour period of treatment. A 14% stimulation of root elongation after 24 hours of treatment with 3-indoleacetic acid at a concentration of 10^{-1} μgm/l and a 10% stimulation with 3-indole acetaldehyde at $10^{-3.2}$ μgm/l were statistically established.

5. Juice obtained from *Artemisia* roots had the capacity to convert 1-naphthalene acetaldehyde to 1-naphthaleneacetic acid. Concurrent with the conversion, however, some of the aldehyde was otherwise changed. 1-Naphthaleneacetic acid was stable in root juice. If the aldehyde has to be converted to acid in order to become active, the conversion must occur within 4 hours in the living root, since the inhibition of elongation observed with the aldehyde showed no increase from 4 to 24 hours.

6. All four substances at relatively high concentrations caused formative effects on roots. Root elongation was virtually stopped, subterminal swellings appeared, and root-hair production was greatly limited. At lesser concentrations, root growth was still strongly inhibited,

and a thick fuzz of root hairs was observed, probably an effect caused by the occurrence of more epidermal cells, and therefore more root hairs, per unit root length.

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RELATION OF HALOGEN POSITION TO PHYSIOLOGICAL PROPERTIES IN THE MONO-, DI-, AND TRICHLOROPHENOXYACETIC ACIDS[1]

J. M. F. LEAPER AND J. R. BISHOP

Introduction

Within the past few years the chlorinated phenoxyacetic acids have become one of the most important new groups of organic chemicals in the United States. This is almost entirely due to the prominence in the herbicide field acquired by 2,4-dichlorophenoxy- and 2,4,5-trichlorophenoxyacetic acids since the first synthesis of these two compounds by POKORNY (**13**) in 1941 and to the subsequent biological work in many independent laboratories here as well as in England and Canada.

The relationship between the position of the chlorine groups in the ring of the substituted phenoxyacetic acids and their hormone-like effects on plant life is of major interest. It is obvious that the various isomers must be of the highest possible purity to insure the specific nature of the physiological effects of each derivative. This is important both for this study and for subsequent experiments which might elucidate unusual biological applications of specific isomers. The mono-, di-, and trichlorophenoxyacetic acids were selected for the present work, since this group included the two apparently outstanding isomers. It is remarkable that at this date there should still remain four of these phenoxy acids unreported in the literature and consequently untested biologically. This work was proposed to fill in these gaps and to obtain preliminary data on "hormone" responses from the new compounds as well as to make tests on the older ones using more highly purified preparations.

Synthesis of compounds

Methods used in synthesis were selected for production of compounds unquestionably free from isomeric impurities. For this reason it was decided that the amides, which are readily convertible into the corresponding acids, should be the first step in the synthesis because they are much more readily recrystallized and purified than are the acids. Also their melting points would be an additional

[1] Contribution from the Agricultural Chemicals Laboratory, American Chemical Paint Co., Ambler, Pennsylvania.

identification characteristic. The acids could then be obtained in a pure state by alkaline hydrolysis of the pure amides with only one recrystallization. As a third criterion the anilides of the acids were also prepared, purified, and their melting points determined.

Since only acetic acid derivatives were involved in this study and not any higher homologues with longer side chains, it was not necessary to follow SYNERHOLM and ZIMMERMAN's method (**17**). They made the acid first by treating the corresponding phenol with ethyl bromacetate and saponification of the ester, then made the acid chloride using thionyl chloride, amidized this with ammonia, and then recrystallized. All this involved the steps: phenol–ethyl ester–acid–acid chloride–amide. It was found more convenient to make the amide directly from the pure phenol by treatment in an alkaline alcoholic solution with pure chloro acetamide. This method was first described by the Berlin Aniline Company in 1899 (**1**), where it was pointed out that the reaction works well in all cases, whereas with certain substituted phenols the ordinary methods fail. This method is applicable only to acetic acid derivatives and not to the higher homologues dealt with by the Boyce Thompson group (**17**).

All the various phenols used in the above synthesis had been previously reported, and, of these, 2-, 3-, and 4-monochlor, 2,4-dichlor, and 2,4,5- and 2,4,6-trichlor were available from the Eastman Kodak Company. The others were made by methods described in the literature, taking care to recrystallize or redistil each one until it had reached the maximum degree of purity. These methods were briefly as follows:

2,3-Dichlor phenol.—M.p. 58. Metachlor phenol was disulfonated to 4,6-disulfonic acid, the barium salt of this chlorinated in the 2 position, and the sulfonic acid groups removed by hydrolysis as described by HODGSON and KERSHAW (**6**).

2,5-Dichlor phenol.—M.p. 58. 2,5-Dichlor aniline was diazotized and boiled with acid (**12**).

2,6-Dichlor phenol.—M.p. 66–68. The ethyl ester of 4-hydroxy benzoic acid was dichlorinated to the 3,5-dichlor compound, and the CO_2 then split off by heating with dimethyl aniline after the ester had been saponified as described by TARBELL and WILSON (**19**).

3,4-Dichlor phenol.—M.p. 64.5. 3,4-Dichlor nitro benzene was reduced to the aniline which was then diazotized and boiled with acid as usual (**7**).

3,5-Dichlor phenol.—M.p. 68. 2,6-Dichlor-4-nitro aniline was diazotized, boiled with alcohol to remove NH_2 and the resulting 3,5-dichlor nitrobenzene reduced, and the aniline diazotized and boiled with acid as usual (**2**).

2,3,4-Trichlor phenol.—M.p. 79. Metachlor phenol was mono-sulfonated to 6-sulfonic acid by the method of HODGSON and KERSHAW (**6**), then dichlorinated to 2,3,4-trichlor-6-sulfonic acid, and the SO_3H group hydrolyzed off.

2,3,5-Trichlor phenol.—M.p. 62. 2,4-Dichlor aniline was acetylated, mononitrated, and hydrolyzed to give 2,4-dichlor-6-nitro aniline. This was converted by the Sandmeyer reaction to 2,3,5-trichlor nitro benzene, which was then reduced to the aniline, diazotized, and boiled with acid following the procedure of HODGSON and KERSHAW (**5**).

2,3,6-Trichlor phenol.—M.p. 58. 2,5-Dichlor phenol was sulfonated with oleum, and the pure barium salt of the 4-sulfonic acid prepared as usual. This was chlorinated with NaOCl to make the 2,3,6-trichlor phenol-4-sulfonic acid

following the technique of HODGSON (6) as used for the 2,3,4-trichlor phenol. The So_3H was then hydrolyzed off. Later another sample of 2,3,6-trichlor phenol, made by hydrolysis of 1,2,3,4-tetrachlor benzene, was obtained from the Hooker Electro Chemical Company. These samples, when converted to their corresponding phenoxy amides and acids, gave products with identical melting points.

3,4,5-Trichlor phenol.—M.p. 101. 2,6-Dichlor-4-nitro aniline was diazotized and converted by the Sandmeyer reaction to 3,4,5-trichlor nitro benzene which was reduced to the aniline, and this was diazotized and boiled as usual to get the phenol as described by TIESSENS (22).

The amides were next prepared from the phenols by the method above mentioned, the same molar proportions being used in all cases, for example:

Phenol	0.1 mole
NaOH 50% solution	0.11 mole
Chloro acetamide	0.11 mole
Isopropanol 99%	60–70 cc.

This mixture was refluxed for 1 hour, poured into 250 cc. of cold water, and the pH value brought to 12.0 by addition of NaOH solution. The amide formed was filtered off, washed, and dried. The yield of amide varied a little in some of the isomers but in general ranged from 60% to 80% of theory. Any unconverted phenol remained in the NaOH solution. The amide thus obtained was in all cases pure enough to give an unchanged melting point on one recrystallization from anhydrous methanol.

The acids were made from the pure amides by boiling with normal NaOH for half an hour, diluting, and filtering off any trace of unchanged amide. The clear solution was precipitated by addition of HCl, and the resulting acid filtered off, washed with water, and dried at 100° C. All phenoxy acids so obtained were recrystallized from benzol or glacial acetic acid once, which was sufficient in all cases to give a product having the theoretical neutralization value and a constant melting point. The anilides were obtained by heating the respective acids with 10% molar excess of redistilled aniline on the hot plate at about 180° C. for 1 hour. The crude anilide was freed from excess aniline by pouring while melted into very dilute HCl, stirring for 10 minutes while still melted, then cooling and stirring until solidified. After filtering the cooled granular material and washing, the precipitated anilide was treated again with warm dilute NaOH solution to dissolve any trace of remaining free phenoxy acid. The filtered-off and washed anilide, after drying, was recrystallized from methanol. Melting points of the various compounds are given in table 1, with notations where the melting points given in the previous literature were appreciably different.

The ortho toluidide derivatives of 2,4- and 3,4-dichlorophenoxyacetic acids were prepared also because the anilides of these two acids had almost the same melting point. The anilide of 2,4,6-trichlorophenoxyacetic acid, which it will be noted has also about the same melting point, would not be mistaken for either of the former two because of the different neutralization value of the parent acid.

Experimentation

It was decided that the method described by MACHT and LIVINGSTON (9) was the most convenient available for determining the comparative biological activity of the foregoing phenoxy acids in a reproducible quantitative way. In this method aqueous solutions of the sodium salts of the acids being tested were mixed with an equal volume of the stand-

ard nutrient solution devised by SHIVE (15) and their effect in causing various degrees of inhibition of the growth of 3-day-old seedlings of *Lupinus albus* was observed. Four strengths of each acid were used—0.1%, 0.01%, 0.001%, and 0.0001%—to ascertain the highest dilution in which a given acid caused growth inhibition. Ten replicates of each dilution were run, and ten roots were run as a control with equal amounts of SHIVE's solution and distilled water. The roots were suspended for the greater part of their length in these solutions for 24 hours in the dark at 20° C. Root length was determined before and after immersion, and amount of growth shown in the solution was calculated as a percentage of the growth attained in the control solution. The "percentage of inhibition" was obtained by subtracting the latter figure from 100.

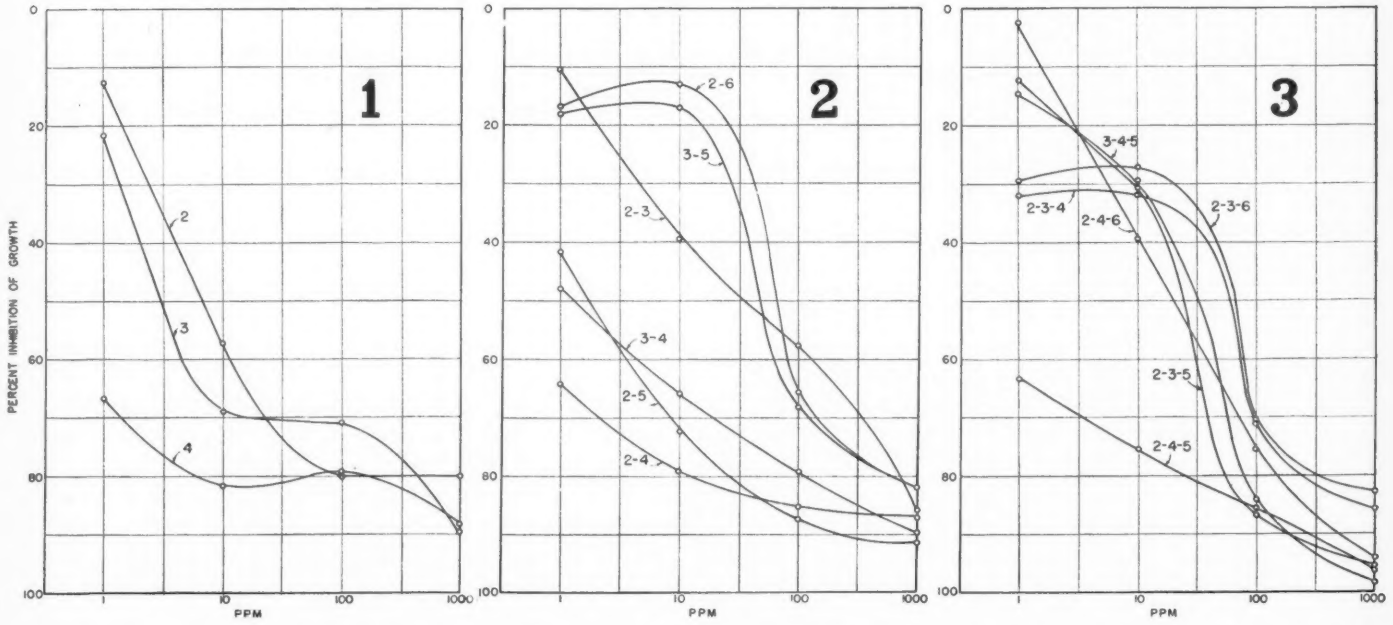
THIMANN (20) pointed out that the inhibition in root length is roughly proportional to the logarithm of the concentration of acid, so it was expected that the data obtained in the present study would be best visualized in a graph with the concentration plotted exponentially against the "percentage of inhibition" (figs. 1, 2, 3). Figure 1 shows that of the monochlorophenoxyacetic acids at a dilution of 1 p.p.m., the 4-chloro isomer was outstanding in inhibiting effect, being roughly four times as effective as the 2-chlor or 3-chlor. At a dilution of 10 p.p.m., however, the three isomers differed much less. In figure 2 the dichloro derivatives fall into two groups, the most inhibitory isomers being the 2-4, 2-5, and 3-4. This is apparent at 10 p.p.m. The 2-3, 3-5, and 2-6 isomers are much less inhibitory. The 2-4 curve shows the most inhibitory effect of all the

TABLE 1

UNCORRECTED MELTING POINTS OF MONO-, DI-, AND TRICHLOROPHENOXYACETIC ACIDS AND DERIVATIVES

Position of chlorine groups in nucleus	Amide	Acid	Anilide	Ortho toluidide
2	150–1	146–7	123–4[a]	
3	*129–30	110–11	*80–1	
4	142[b]	159–160[c]	127.5–128.5[d]	
2,3	*172.5–173.5	*174–175	*106–107	
2,4	155–6[e]	140–1	111–12	*138–9
2,5	*140–1	148–9[f]	*145–145.5	
2,6	*146–7	137–8[g]	*86–87	
3,4	*151.5–152.5	141–2	*111.5–112	*126–7
3,5	*175–6	117.5–118.5	*155–6	
2,3,4	*198–198.5	171–2[h]	*143–4	
2,3,5	*170.5–171.5	*164–5	*185.5–186.5	
2,3,6	*139–140	*148–9	*87–88	
2,4,5	174.5–175.5	158–9[i]	178–9	
2,4,6	197.5–198.5	187–8[j]	*110.5–111.5	
3,4,5	*188–9	*150–1	*164–5	

* Not previously reported.
[a] MINTON and STEPHEN (10) give 121.
[b] MINTON and STEPHEN (10) give 133.
[c] HAYES and BRANCH (4) give 156.7–157.2.
[d] MINTON and STEPHEN (10) give 125.
[e] SYNERHOLM and ZIMMERMAN (17) give 152–153.
[f] SYNERHOLM and ZIMMERMAN (16) give 142–143.
[g] TARBELL and FANTA (18) give 134.5–135.
[h] SYNERHOLM and ZIMMERMAN (16) give 150–160.
[i] POKORNY (13) gives 153.
[j] SYNERHOLM and ZIMMERMAN (16) give 190.



FIGS. 1–3.—Relation between inhibition of root growth of *Lupinus albus* and concentration of (fig. 1) monochlorophenoxyacetic acids, (fig. 2) dichlorophenoxyacetic acids, and (fig. 3) trichlorophenoxyacetic acids.

isomers at a dilution of 1 p.p.m. In figure 3 the striking difference between the 2-4-5 isomer and all the other five is evident at dilutions of 10 p.p.m. or less.

TABLE 2

APPROXIMATE MINIMUM THRESHOLD CONCENTRATIONS (MG.) OF PHENOXYACETIC ACIDS PER GRAM OF LANOLIN FOR RESPONSE IN TOMATO TEST

Position of chlorine groups in nucleus	Cell elongation (epinasty and other growth curvatures)	Formative effects (modification of organs)	Rooting on intact tomato stems
2	0.5	0.25	2.5
3	0.1	Inactive	2.0
4	0.1	0.06	1.0
2,3	1.0	Inactive	5.0
2,4	0.015	0.003	0.32
2,5	0.01	Inactive	0.25
2,6	Inactive	20.0	Inactive
3,4	0.05	0.50	2.50
3,5	Inactive	5.0	Inactive
2,3,4	1.0	1.0	Inactive
2,3,5	Inactive	Inactive	Inactive
2,3,6	Inactive	Inactive	Inactive
2,4,5	0.025	Inactive	0.1
2,4,6	Inactive	Active	Inactive
3,4,5	Inactive	5.0	Inactive

Parallel tests also were conducted on the same series of acids under the direction of Dr. P. W. ZIMMERMAN at the Boyce Thompson Institute using his well-known techniques (**24**). Though some of the acids listed had been previously tested by these methods, the tests were repeated for tabulation with the four new acids included as well as for more highly purified samples of those acids which had been reported earlier. Most of the amides and anilides in table 1 have not been reported before, but this paper is concerned only with biological tests on the acids themselves. The tests conducted at the Boyce Thompson Institute were based on responses of tomato plants to lanolin applications containing the chemicals dissolved in the stated concentration (table 2).

In table 3 the threshold concentrations for cell elongation and rooting are rearranged, using reciprocals of the mg/gm figures given in table 2 so that the numbers indicate direct efficiency for the stated biological effects per unit of weight of the acid. The most efficient compound in column 2 is rated at 100, as in the work of THOMPSON *et al.* at Camp Detrick (**21**).

If from table 3 we consider only relative efficiencies of 10% or over of the maximum—which happens to be 2,5-dichlorophenoxyacetic acid in this series of tests—we eliminate all but the 2-, 3-, and 4-monochloro-, the 2,4-, 2,5-, and 3,4-dichloro-, and the 2,4,5-trichloro-compounds. These are the same compounds which were outstanding in the *Lupinus* root-inhibition tests (figs. 1, 2, 3). They are the only derivatives of the series of mono- and poly-chlorophenoxyacetic acids showing relatively high potency in stimulating cell elongation or rooting in tomato stems when tested by the lanolin application method, and in inhibiting root growth of *L. albus*.

TABLE 3

RELATIVE "HORMONE" EFFICIENCY OF CHLOROPHENOXYACETIC ACIDS IN TOMATO TEST

Position of chlorine groups in nucleus	In cell elongation (epinasty, etc.)	In rooting on intact stems
2	2.0	10.0
3	10.0	12.5
4	10.0	25.0
2,3	1.0	5.0
2,4	66.6	77.5
2,5	100.0	100.0
2,6	0	0
3,4	20.0	10.0
3,5	0	0
2,3,4	1.0	0
2,3,5	0	0
2,3,6	0	0
2,4,5	40.0	250.0
2,4,6	0	0
3,4,5	0	0

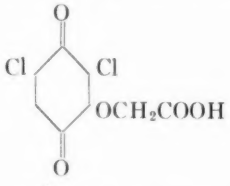
Discussion

Despite the many difficulties in evaluating plant growth substances by diverse methods, as pointed out by VELDSTRA (23) and others, remarkably close agreement exists between results obtained by the lanolin method on tomato plants and those derived by the *Lupinus* technique if selection is made of the inhibition figures obtained at 10 p.p.m. concentration (figs. 1, 2, 3). Although effects of the 2- and 3-monochlorophenoxyacetic acids diverge unless a critical concentration of 10 p.p.m. is adhered to, it is noteworthy that results from the *Lupinus* technique which uses roots completely immersed in the test solution should so closely parallel results of the lanolin-salve application tests of ZIMMERMAN where the effects observed are partly dependent on transportation of the active material through plant tissue. It is probable that for all these closely related phenoxy acids the transportation factor varies little with halogen positions so that the results obtained may be considered as criteria of primary activity of the various acids.

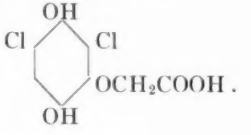
If the structural formulas of the seven halophenoxy acids with the highest potency are portrayed as shown in the formulas at the bottom of the page, where $R = CH_2COOH$, it is readily seen that among all fifteen acids these are the only ones possible which have two unsubstituted positions in the ring which are para to each other. The original postulation of KOEPFLI *et al.* (8) was that for biological activity a chemical should have an unsaturated ring system with a side chain having a carboxyl group connected to the ring through at least one carbon atom. MUIR *et al.* (11) and also HANSCH and MUIR (3) suggested an additional structural requirement—that the compound should have a free ortho position. With regard at least to the restricted class of chlor-substituted phenoxy acids (and there is good evidence for including other halogen-substituted phenoxy acids also), it would seem that if high biological potency only is considered, an additional requirement would therefore be that there should be another free position on the benzene nucleus opposite to the free ortho position above postulated.

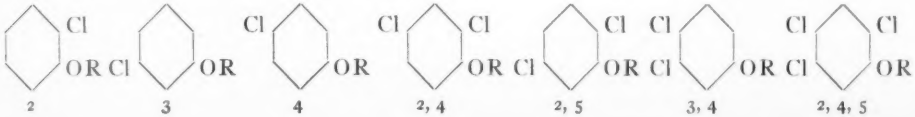
This structural requirement could be explained by assuming that in plant metabolism these compounds are involved in the formation of derivatives having a quinonoid structure. Such quinonoid derivatives can be readily visualized as taking part in the oxidation-reduction systems that are usually associated with growth.

Thus, 2,4-dichlorophenoxyacetic acid, for example, might first become oxidized to 2,6-dichloro-3-glycollic acid quinone,



which in turn could become reduced to the corresponding quinol





2 3 4 2, 4 2, 5 3, 4 2, 4, 5

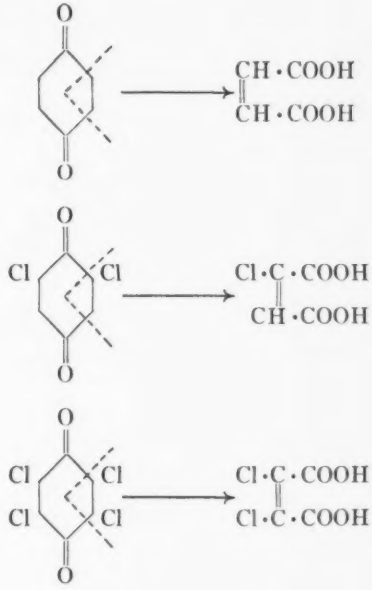
The toxicity of either or both of these compounds, when formed in the life-processes of the plant at whatever point the transported 2,4-D might reach, could be the determining factor in causing injury or death of the organism. This can best be proved by synthesis of such quinones and hydroquinones in the laboratory and making direct tests with them. Work on this phase of the problem is in progress.

It is interesting in this connection that 2,6-dimethoxy benzoquinone is known to be a powerful inhibitor of the growth of *Staphylococcus aureus*, although this may not necessarily be the case with higher forms of plant life. Whether the effects are to be ascribed to the methoxy groups or to the quinone structure in this case has not been determined. The well-known inhibitory effect of chloranil and related quinones on fungus growth may also be relevant. Here the nuclear chlorine as well as the quinone structure are no doubt important.

Another possible contingency that arises from postulation of a quinone formation would be that the molecule may be subjected to a further degradative oxidation. For instance, the above-mentioned quinone derived from 2,4-D could be visualized as breaking down by further oxidation to chloromaleic acid, just as benzoquinone itself gives rise to maleic acid and chloranil to dichloromaleic acid, as shown in next column.

The latter possibility would seem to connect the phytocidal properties of the seven outstanding halophenoxy acids above selected with the maleic acid derivatives having strong herbicidal properties recently described by SCHOENE and HOFFMANN (**14**).

The preceding analyses are admittedly speculative, and there may be quite different reasons why the optimum herbicidal structure appears to involve the presence of the two opposite unoccupied positions. Nevertheless, the most effective compounds have this structure, and the above hypothesis seems as apt as any other.



Summary

1. All the mono-, di-, and trichlorophenoxyacetic acids have been prepared in the highest possible purity, and their melting points have been recorded. Their corresponding amides and anilides have also been made, and their melting points recorded. Four of these acids, ten of the amides, and eleven of the anilides have not previously been reported.

2. All these chlorophenoxyacetic acids, including the four not hitherto tested, have been subjected to selective "hormone" screening tests with tomato plants using the procedure of ZIMMERMAN and HITCHCOCK (**24**) at the Boyce Thompson Institute and also to the root-inhibition test of MACHT and LIVINGSTON (**9**) using *Lupinus albus*.

3. The highest physiological activity of chlorophenoxyacetic acids has been shown to be associated with the presence of two unsubstituted positions in the benzene ring para to each other.

4. The possibility of the formation of compounds having quinonoid structure being connected with their maximum herbicidal potency is discussed.

AMBLER, PENNSYLVANIA

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SALT TOLERANCE AND CHEMICAL COMPOSITION OF RHODES AND DALLIS GRASSES GROWN IN SAND CULTURE[1]

H. G. GAUCH[2] AND C. H. WADLEIGH[3]

Introduction

Rhodes grass (*Chloris gayana* Kunth.) and Dallis grass (*Paspalum dilatatum* Poir.) show considerable promise for use in irrigated pastures in southwestern United States. Field observations suggest that these species have moderate salt tolerance, but little quantitative information is available. It is generally believed that when Rhodes grass is grown on saline soils, sufficient quantities of salt are accumulated in the herbage so that it acts as a cathartic when eaten by livestock (**21**).

It was deemed advisable to study the salt tolerance of these two species under controlled conditions and to ascertain the influence of salinized substrates on their mineral composition.

Material and methods

The plants were grown in 5-gallon crocks of sand each of which was automatically supplied at hourly intervals with ½ gallon of solution from an accompanying 5-gallon reservoir of a specified nutrient solution (**15**). The base nutrient solution (control) had the following composition, expressed in milliequivalents per liter (m.e./l.): Ca, 6.4; Mg, 5.3; Na, 1.8; K, 2.4; Cl, 1.4; SO_4, 5.6; NO_3, 7.6; and H_2PO_4, 0.8. Boron and manganese were each added at 0.5 p.p.m., and iron at 1.0 p.p.m. supplied as ferric tartrate. The treatments, listed in table 1, were randomized within blocks with three replicates of each treatment.

TABLE 1

LIST OF TREATMENTS AND PERTINENT CHARACTERISTICS OF SOLUTIONS

Treatment	M.e. of added salt per liter	Osmotic pressure of solution (atm.)
Base nutrient		0.5
$+Na_2SO_4$	72	2.5
$+NaNO_3$	48	2.5
$+NaCl$	48	2.5
$+CaCl_2$	70	2.5
$+MgCl_2$	33	1.5
$+NaHCO_3$	12	0.8

Seed was supplied by Dr. O. S. AAMODT of the Division of Forage Crops and Diseases, U.S.D.A., Washington, D.C. The species and serial numbers were: Rhodes grass (*Chloris gayana* Kunth.), F.C. #22,754; and Dallis grass (*Paspalum dilatatum* Poir.), F.C. #29,724.

Germination of "seeds" of both species was facilitated by rubbing them between sheets of sandpaper (**27**) to remove lemmas and paleas. Several hundred 3-inch clay pots, filled with equal parts of Pachappa loam and peat moss, were planted on July 17, 1944, to each of these species so as to provide for selection of uniform experimental plants. Rhodes

[1] Contribution from the U.S. Regional Salinity and Rubidoux Laboratories, Riverside, California, Bureau of Plant Industry, Soils and Agricultural Engineering, Agricultural Research Administration, U.S. Department of Agriculture in co-operation with the eleven Western States and the Territory of Hawaii.

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grass seedlings were transplanted to the sand cultures on August 7, and the Dallis grass seedlings on August 17. Their roots were washed nearly free of soil before transplanting. Differential treatment for Rhodes grass was initiated by adding one-half the final concentration of added salines on August 23, and bringing to full strength the following day; for Dallis grass, initiated September 8, and brought to full strength September 9.

The runners on Rhodes grass were clipped and analyzed separately from the rest of the tops. The chemical analyses on both species were made on the first clippings, each grass being first clipped 13 days after the salt treatments were initiated. The clippings were dried in a forced-draft oven at 80° C. for 24 hours and then ground in a small Wiley mill to pass a 40-mesh sieve. An aliquot of a given sample was wet-ashed with nitric and perchloric acids, and REITEMEIER's methods (**28**) were followed for the determinations of calcium, potassium, magnesium, sulfur, and phosphorus. Sodium was determined gravimetrically by the uranyl zinc acetate method, following certain precautions suggested by BROADFOOT and BROWNING (**7**) to avoid interference from potassium and phosphorus. Chloride was determined by a modification of the method of CLARK *et al.* (**9**).

Results and discussion

GROWTH AND APPEARANCE OF PLANTS

Growth of Rhodes grass was luxuriant, but the Dallis grass tended to be chlorotic from time to time, and additional ferric citrate was occasionally added to the surface of the sand cultures to counteract this tendency. Chlorosis frequently occurs on Dallis grass even under field conditions, however, and disappearance of chlorosis is sometimes used as an indication of the need for irrigation. The conditions which bring on chlorosis under field conditions are unknown, but it is frequently noted that high soil moisture content is conducive to the development of this symptom in many species (**37**).

It is to be noted (fig. 1) especially that the presence of 12 milli-equivalents of $NaHCO_3$ per liter of culture solution had no effect on growth of Rhodes grass. By contrast, addition of 1 atmosphere osmotic pressure (1 atm. OP) of $MgCl_2$ (33 m.e./l.) to the culture solution proved to be quite inhibitive to growth of this species; much more so than the addition of 2 atm. OP of the other neutral salts. Though Rhodes grass is relatively salt tolerant, it appears to be just as sensitive to $MgCl_2$ as nontolerant Red Kidney beans (**16**). There tended to be a slight reduction in growth of Rhodes grass when other neutral salts were added to the nutrient solution to produce 2 atm. OP. This tendency was definite, however, only when both component ions of a salt were monovalent, but this distinction is probably fortuitous rather than real. On the average, the cultures of Rhodes grass with 2 atm. OP of added salt yielded approximately 85% as much as the controls. This is indicative of good salt tolerance.

For Dallis grass the yields of all salinized cultures were well below that of the controls. The most striking difference in the responses of the two grasses was brought about by the addition of 12 m.e./l. of $NaHCO_3$ to the base nutrient solution: whereas Rhodes grass was unaffected by this treatment, Dallis grass was markedly sensitive to it (figs. 1, 2). A severe chlorosis of Dallis grass was induced by $NaHCO_3$ and could not be corrected by an intensified supply of ferric citrate to the sand cultures. The plants

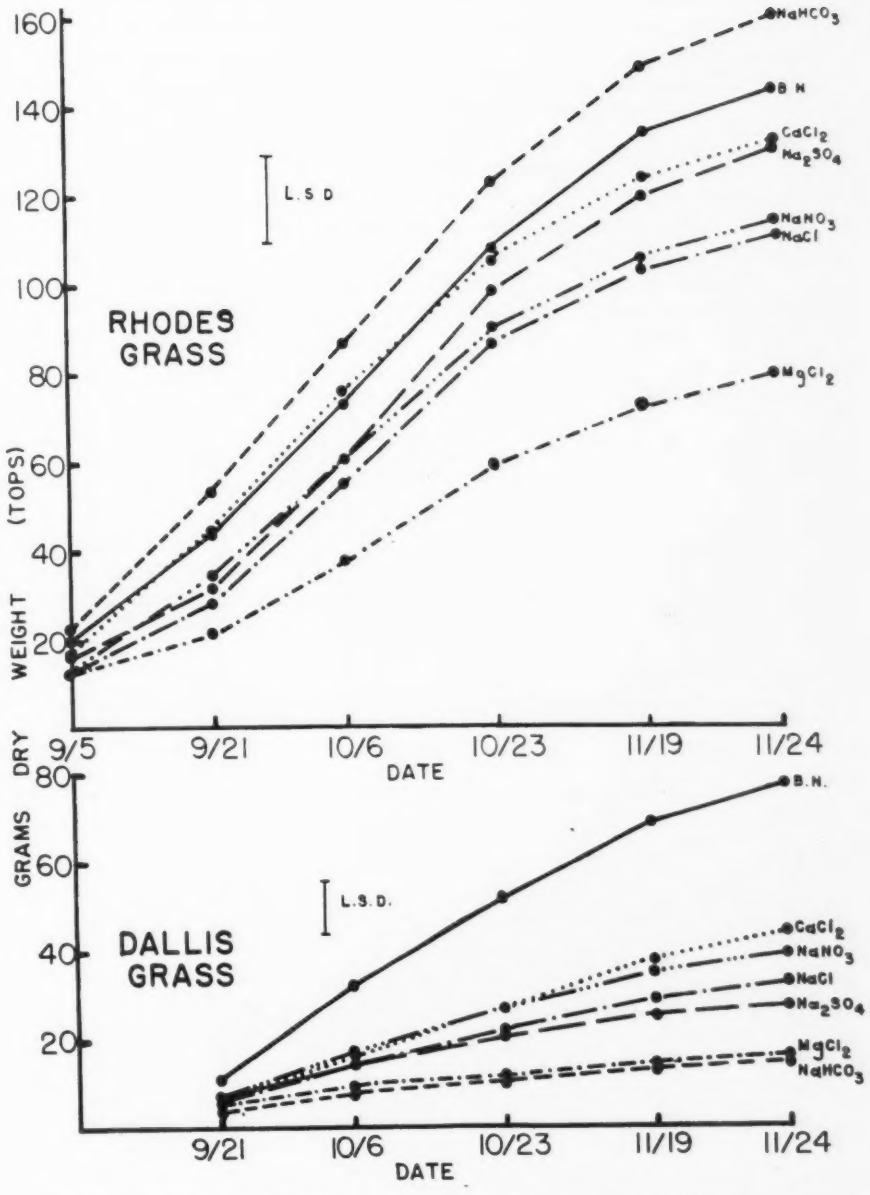
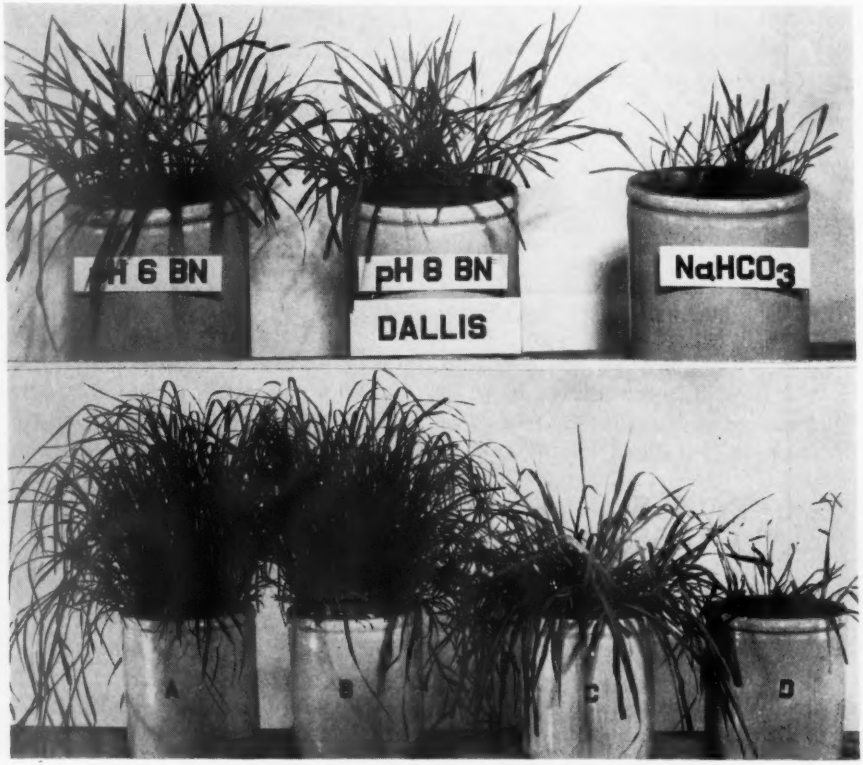


FIG. 1.—Effect of high concentrations of various salts in base nutrient solution on growth of Rhodes and Dallis grasses.

became so seriously affected that they were nearly dead by the end of the experiment.

The following year, 1945, an auxiliary experiment was carried out to determine whether the response of Dallis grass to $NaHCO_3$ was primarily related to the relatively high pH values associated with the presence of $NaHCO_3$. In order to distinguish between the effect of high pH per se from the high pH brought about by added $NaHCO_3$, a base nutrient with a pH value of 8.0, adjusted by added NaOH, was included in the test. As is illustrated (fig. 3), the deleterious effect of $NaHCO_3$ on Dallis grass cannot be attributed to high pH alone; this grass appears to have a special sensitivity to the bicarbonate ion. On the other hand, the insensitivity of Rhodes grass to $NaHCO_3$ was also noted by CLARK (8), who reported that in Arizona it made "a fine healthy growth where black alkali is so strong that all other crops, save Australian saltbush, have failed to grow." A "black alkali" soil frequently contains relatively high amounts of soluble carbonate and bicarbonate. STOUT *et al.* (32) stated that bicarbonate is not accumulated as such by plants and that the



FIGS. 2, 3.—FIG. 2 (*below*), growth and appearance of Rhodes (*A*, *B*) and Dallis (*C*, *D*) grasses receiving base nutrient solution (*A*, *C*) or base nutrient containing, in addition, 12 m.e. of $NaHCO_3$ per liter (*B*, *D*). Fig. 3 (*above*), effect of pH of base nutrient solutions and effect of 15 m.e. of $NaHCO_3$ added per liter of pH 6 base nutrient solution on growth and appearance of Dallis grass.

small amounts of HCO_3 that are absorbed are subsequently reduced to compounds of lower valence.

One atmosphere of added $MgCl_2$ was even more toxic to Dallis than to Rhodes grass: their relative yields on this treatment were 22% and 55% of their respective controls.

The addition of 2 atm. OP of other neutral salts—NaCl, Na_2SO_4, $NaNO_3$, or $CaCl_2$—to the culture solution definitely limited the growth of Dallis grass, but there was little or no specific effect for the four salts studied. The yield following such salinization averaged only 51% of that of the controls, indicating an appreciably lower degree of salt tolerance for this species than for Rhodes grass.

Since isotonic concentrations of these four salts did not bring about definite differences in growth response for the two grasses, it is concluded that the effect of these saline cultures on growth took place largely as a result of the decreased physiological availability of water. The chemical analyses were undertaken partially to evaluate this conclusion.

The degree of succulence of the two species was very similar in the control treatments, but on salinized cultures the percentage of dry matter in Dallis grass was consistently higher than for Rhodes grass (table 2). In line with the observation that $NaHCO_3$ had little effect on the growth of Rhodes grass, the succulence of its herbage from this treatment was virtually identical with that from the control cultures. Other saline treatments effected a moderate increase in percentage of dry matter in Rhodes grass; and all saline cultures resulted in a comparatively high percentage of dry matter in Dallis grass. This effect on Dallis grass appears to be related to its retarded growth on the salinized nutrient solutions. Correspondingly, the greater the inhibition of growth of barley on saline cultures, the higher was the percentage of dry matter in the tops (**14**). Generalizations from these observations should be developed with caution, however, as HAYWARD and WADLEIGH (**20**) pointed out; the effect of salinity on succulence may be diametrically opposite at low and high levels of salinity, and saline substrates may effect an increase, decrease, or no response at all on percentage of dry matter, depending on the salts and species involved.

TABLE 2

AVERAGE PERCENTAGE OF DRY MATTER IN HERBAGE (DATA FROM ALL HARVESTS POOLED)

Treatment	Rhodes grass	Dallis grass
Control	12.3	11.9
$NaHCO_3$	12.5	15.9
NaCl	13.6	15.2
$NaNO_3$	13.2	13.6
Na_2SO_4	14.4	16.6
$CaCl_2$	14.5	15.8
$MgCl_2$	14.3	17.0

The relationship between effects on percentage of dry matter and the prevalence of guttation is of interest. There were frequently copious amounts of guttated water on both grasses on the control cultures and on the Rhodes grass receiving added $NaHCO_3$. All other treatments (salt cultures) failed to show guttation at any time during the course of the experiment. The correspondence of increase in percentage dry matter with failure to guttate would seem further to indicate that the saline treatments impeded entry of water.

ELEMENTAL COMPOSITION

The first clippings were used for chemical analyses. The runners of the Rhodes grass plants were harvested and

analyzed separately to determine whether the composition of this fraction varied from that of the tops in general, but there were no outstanding differences between the concentrations of a given element in the leaves and the runners of Rhodes grass produced on a given treatment (table 3).

CALCIUM.—On the average, Dallis grass was found to contain about 50% more calcium than Rhodes, and a differential in content was evident under every experimental treatment. The data obtained for calcium content of Dallis grass are relatively high in comparison with values of 16.4–34.2 m.e./100 gm. in young Dallis grass from the Gulf Coast Prairie (**13**), and values of 25.7–42.8 m.e./100 gm. when grown on Everglades peat (**24**) that tends to favor comparatively high accumulations of calcium in plants. The $CaCl_2$ treatment induced

TABLE 3

EFFECT OF VARIOUS TREATMENTS ON ELEMENTAL COMPOSITION OF RHODES AND DALLIS GRASSES

TREATMENT	M.E./100 GM. DRY MATTER						
	Ca	Mg	K	Na	Cl	SO_4*	H_2PO_4†
	Rhodes grass (minus runners)						
Control	36.5	28.0	90.0	49.7	10.4	27.0	18.9
+NaCl	20.6	19.9	67.6	99.9	25.8	22.1	17.1
+Na_2SO_4	14.4	15.3	55.9	121.8	10.9	42.2	16.2
+$NaNO_3$	19.6	20.4	79.6	94.2	7.0	25.0	18.9
+$NaHCO_3$	19.4	17.8	59.2	93.1	12.2	25.6	12.4
+$CaCl_2$	49.4	7.6	101.6	38.8	43.4	18.6	13.1
+$MgCl_2$	13.4	58.6	83.3	39.6	39.6	22.0	17.9
	Rhodes grass (runners only)						
Control	33.7	20.4	110.8	36.6	19.4	19.1	17.4
+NaCl	17.6	16.5	79.6	102.1	52.9	14.3	17.3
+Na_2SO_4	14.5	19.1	69.6	113.6	14.1	31.3	17.4
+$NaNO_3$	17.0	19.1	65.0	89.9	11.3	16.0	16.6
+$NaHCO_3$	20.3	17.8	62.1	86.1	21.5	23.5	13.5
+$CaCl_2$	36.2	10.2	110.8	37.1	50.7	19.5	12.7
+$MgCl_2$	16.8	53.6	89.1	30.9	40.7	14.8	17.2
	Dallis grass						
Control	48.5	30.3	136.6	3.2	8.8	32.9	18.0
+NaCl	31.1	26.8	71.7	19.6	18.6	29.0	14.7
+Na_2SO_4	25.6	24.2	71.3	31.0	5.3	34.5	14.7
+$NaNO_3$	32.3	28.0	72.1	22.1	6.3	23.5	14.5
+$NaHCO_3$	31.9	38.8	79.9	16.3	16.0	17.1	9.0
+$CaCl_2$	61.4	13.0	89.0	3.5	30.1	21.4	11.9
+$MgCl_2$	34.6	68.8	81.6	3.0	21.4	26.4	13.9

* Sulfur, as SO_4^{--}.

† Phosphorus, as $H_2PO_4^-$.

very high levels of calcium in the foliage of the two grasses. This accumulation appeared to be in no way related to growth response, however, since yields for each grass on the $CaCl_2$ cultures were virtually identical with yields under other saline treatments that induced relatively low levels of calcium in the herbage. For example, the Na_2SO_4 treatment effected the lowest levels of calcium observed for each species, and yet the growth response was not significantly different from that of cultures with other neutral salines at isosmotic pressures. Added $MgCl_2$ also appeared to depress entry of calcium into the plants, with the effect being more pronounced in Rhodes than in Dallis grass, even though impairment of growth was much more serious in the Dallis grass on this treatment. This response in the $MgCl_2$ cultures was evidently not related to an inadequacy of calcium in the tissues, since excellent growth of both species may be observed when the foliage contains the respective levels of calcium observed under the $MgCl_2$ treatment. The data emphasize the wide variation in concentration of the calcium ion which may be found in plant tissue without having any evident relation to growth response.

MAGNESIUM.—Dallis grass invariably was found to contain more magnesium than Rhodes. Since a comparable distinction between the two species also prevailed for calcium, it appears that the divalent ions accumulate with greater facility in Dallis than in Rhodes grass. The $MgCl_2$ treatment induced high concentrations of magnesium in these two grasses. Such accumulation of magnesium appears to be directly associated with the poor growth observed, the more seriously affected Dallis grass having the higher content. The $NaHCO_3$ treatment that was so detrimental to Dallis grass also induced an accumulation of magnesium in the tissue, but the data do not warrant implying a causative relationship in this instance.

The $CaCl_2$ treatment brought about relatively low levels of magnesium in the tissue of both grasses. Their comparative growth response in these cultures, however, renders unlikely any possibility that such levels of magnesium approached inadequacy and became limiting to growth. The data indicate that the concentration of magnesium in the herbage of these two grasses may vary widely without being evidently associated with growth response. On the other hand, plants can readily accumulate magnesium to a concentration at which growth processes are adversely affected.

POTASSIUM.—Culture in nutrient solution is frequently conducive to excessive accumulation of potassium by plants. As a case in point, the control Dallis grass was found to contain 136.6 m.e. of potassium per 100 gm. dry matter; whereas BLASER and STOKES (3) reported the potassium content of this species under fertilizer trials on a Plummer fine sand as varying from 35 to 61 m.e./100 gm. dry tissue. When grown on the base nutrient solution, Dallis was found to have a much higher concentration of potassium in the tops than did Rhodes grass, but added sodium salts definitely limited the accumulation of potassium in both species to the extent that the contents of this element in the two grasses under respective sodium treatments were quite similar. The potassium content was higher in the plants from the $CaCl_2$ treatment than in those from any other salt treatment, and in Rhodes grass this saline induced the highest potassium accumulation observed. VIETS (33) found that calcium is particularly

effective for stimulating potassium absorption, and PIERRE and BOWER (**26**) concluded that potassium absorption by plants is usually decreased by the presence of high concentrations of other cations but may be increased. The latter authors pointed out further that other ions affect potassium absorption less than potassium concentration affects the absorption of other ions, particularly calcium and magnesium.

A comparison of the potassium contents of Rhodes grass from the Na_2SO_4 and $CaCl_2$ treatments is indicative of the wide variation that can take place in the concentration of this element in plant tissue without being associated with growth response. The content of potassium in Dallis grass from the control cultures was much higher than that from salinized cultures, and growth response showed the same general differential; but in the absence of any supporting evidence the authors are inclined to regard this concomitance as fortuitous. In fact, it is possible that the control plants contained excessive potassium for optimum growth and that this condition was conducive to the chlorotic symptoms that tended to develop on the Dallis grass. Corn plants grown in nutrient solutions frequently become chlorotic (**35**), and there is usually an associated tendency for excessive accumulations of bases, especially potassium (**36**).

SODIUM.—The most striking difference in the elemental composition between these two grasses was observed for sodium. COLLANDER (**11**) has published extensive data to substantiate his conclusions that some species accumulate more sodium than others and that the proportion of the total cations represented by sodium is characteristic for a given species. The much greater facility with which sodium accumulates in Rhodes than in Dallis grass is evident (table 3).

The base nutrient solution was made up with tap water which contained 1.8 m.e. of sodium per liter, yet Rhodes grass plants accumulated sodium to the extent of 50 and 37 m.e./100 gm. dry matter in the tops and runners, respectively. By contrast the tops of Dallis grass contained only 3.0 m.e./100 gm. dry matter. The presence of any of the four sodium salts nearly doubled the concentration of sodium in the dry matter of Rhodes grass over that of the control plants. On a relative basis the sodium content of Dallis grass was increased more by the sodium salt treatments than was that of Rhodes (i.e., from 3.0 to around 20 m.e./100 gm. dry matter in Dallis), but the actual concentrations in Dallis were still only one-fourth to one-fifth as high as in Rhodes grass (table 3).

The $MgCl_2$ and $CaCl_2$ treatments had very little if any effect on the sodium content of the plants.

There is a possibility that the roots of Dallis grass may have had considerably higher percentages of sodium than the tops. COLLANDER (**10**) observed this to be the case in most of the sixteen species of plants he studied, and BOWER and WADLEIGH (**4**) found that there was a pronounced accumulation of sodium in the roots of Dallis grass, whereas in Rhodes grass sodium accumulated more in the tops. On salinized cultures, pronounced accumulations of sodium have also been found to occur in the roots of kidney-bean plants (**17**) and peach trees (**19**), even though virtually no accumulation of this element was found in the leaves of these species.

The growth of Rhodes grass was obviously independent of variations in content of sodium within the limitations

of the values observed. Furthermore, the data do not justify any implication that the sodium ion might be either essential or beneficial to the growth of this species. The data in table 3 provide some indication that depressed growth of Dallis grass in cultures salinized with sodium salts might be associated with an abnormally high accumulation of sodium in the tissue; but the fact that isotonic cultures of $CaCl_2$ and NaCl were conducive to virtually identical degrees of growth response, regardless of wide disparity in sodium content, tends to mitigate any idea of a specifically adverse effect of the sodium ion in this grass. In other words, the effect of added sodium in the culture solution on these grasses over and above the intensified physiological scarcity of water effected by the increased osmotic pressure appears to be nil.

CATIONIC INTERRELATIONSHIPS.—There is a prevailing tendency to interpret plant behavior as conditioned by mineral nutrition in terms of a concept of "balance." SHIVE (**30**) presented a good discussion of this viewpoint and stated that it is a goal toward which investigations in mineral nutrition of plants should be directed but that "it is a scientific ideal, however, which with our present knowledge, is impossible of attainment, even under the most rigid system of controls, because it is not possible as yet to isolate all the factors that influence the plant and to deal with them appropriately." Observations on the specific effect of various cations and/or cationic ratios on the physicochemical properties of protoplasm (**29**) and the activity of enzyme systems (**6**) provide a fundamental approach to ascertaining the role of many mineral nutrients in plant metabolism. The results of such studies may be extrapolated toward supporting the concept of "balance" (**34**). The term may be defined as "a combination of factors, elements, or the like—such that the proportions are correct for a certain purpose." In evaluating the role of ionic proportions in plant tissues toward conditioning plant behavior, it is well to keep in mind the note of caution set forth by GOODALL and GREGORY (**18**). They pointed out that if plant behavior is primarily determined by ionic ratios, rather than absolute concentrations, then a given criterion of performance will be constant for a constant ratio regardless of wide variations in the magnitudes of the values entering into the ratio. This has never been demonstrated.

Considerable evidence has accrued that a summation of the cation equivalent values for a given species tends to be a constant under standardized environmental conditions even though mineral nutrition varies widely (**39**). Exceptions to this are occasionally recorded, however (**40**). The data in table 3 indicate that the cation-summation-values for Rhodes grass leaves under the various treatments were fairly constant, varying from 190 to 214 m.e./100 gm., with an average of 202. Thus, variations in concentration of one cation in the leaves were largely compensated for by variations in other cations.

In Dallis grass the cation-summation-values varied from 149 to 219 m.e./100 gm. and certainly may not be regarded as constant. The control plants of this species made the most growth and had the highest value for cation summation, but otherwise there was no evidence for any relationship between total accumulation of cations and growth. In fact, such a relation should not be expected. It appears that Dallis grass can accumulate excessive quantities of potassium as indicated by data for the control cultures. Its much lower potassium content on salin-

ized cultures was not compensated for by accumulations of other cations. Thus, a species characterized by a marked capacity to exclude or accumulate certain cations selectively may be poor experimental material with which to demonstrate constancy of cation-equivalent summations.

All possible cationic ratios were calculated, but there were few instances in which variation in a given ratio appeared to be associated with growth. These few could be more adequately interpreted on the basis of absolute levels of a given cation. For example, the ratio of monovalent to divalent cations (K + Na)/(Ca + Mg) varied from 1.70 to 5.98 in the leaves of Rhodes grass, and from 0.82 to 2.05 in Dallis grass. The variability was quite unrelated to growth, excepting that the lowest values in each grass were observed for cultures receiving added $MgCl_2$ and suffering from magnesium toxicity. Any ratio involving magnesium had unique values for the $MgCl_2$ cultures. Yet, the poor growth of both grasses is explainable on the basis that the absolute accumulation of magnesium in the tissue attained toxic levels. In order to conclude that the ratio of magnesium to other cations was a consideration in the plants afflicted with magnesium toxicity, one would have to demonstrate that given ratios involving magnesium were associated with the same plant response at lower absolute levels of magnesium accumulation.

LINDNER and HARLEY (**22**) found relatively high values for the K/Ca ratio in chlorotic apple and pear leaves; yet, in the present study, the nonchlorotic Dallis grass produced by the NaCl cultures had virtually the same K/Ca ratio as the severely chlorotic herbage from the $NaHCO_3$ cultures. It is not to be implied that the discussion herein is presented as a refutation of the concept of "balance." The effect of levels of one nutrient conditioning the required level of another is too well established (**5**). This approach merely did not prove advantageous in evaluating the data reported.

The poor growth of Dallis grass on cultures receiving added $NaHCO_3$ was the most striking response observed in this experiment, but the data on cation content (table 3) provide no clue to the physiological disturbances involved, and the calculation of ratios gave no further insight.

SULFATE.—As would be expected the Na_2SO_4 treatment brought about an increase in the sulfur content. The most surprising feature, however, was its small order of magnitude in both grasses, less than 50% in Rhodes and around 15% or less in Dallis grass. Other salt treatments had very little effect on the sulfur content of the plants. In this connection barley plants have also been reported to exclude sulfate to a marked degree even though there were 200 m.e. of this ion per liter of substrate (**14**).

CHLORIDE.—In those cultures which did not receive added chloride salts the concentration of chloride was about the same in the two grasses. When such salts were added in rather large amounts, the concentration of chloride in dry matter was increased more in Rhodes than in Dallis grass; for any given treatment, the concentration in Dallis was roughly half as high as in Rhodes grass. The $CaCl_2$ treatment effected the highest accumulation of chloride in both grasses. This effect of a predominance of the calcium cation on chloride absorption is typical (**23**). In fact, the relationship is so pronounced that a $CaCl_2$ culture solution similar to the one used herein has been found to be lethal or extremely deleterious to drupaceous fruit trees as a consequence of excessive chloride toxicity; whereas an isosmotic solution with added

NaCl had comparatively little effect upon the trees (**38**). Nevertheless, the evidence at hand from a comparison of various treatments indicates that the highest accumulations of chloride found in these grasses were not specifically inhibitive to growth.

PHOSPHATE.—The two grasses had very similar concentrations of phosphorus in the dry matter, and, in general, added salts had little effect on this value. Others have noted that the percentage of phosphorus in plants is very little affected by saline substrates (**12, 14**). There were two added salts, however, which lowered the phosphate content of both grasses: $CaCl_2$ and $NaHCO_3$. However, even the low concentration of phosphorus observed for Dallis grass on the $NaHCO_3$ treatment is as high as that found by BLASER and STOKES (**3**) in this species on a sandy soil heavily supplied with phosphatic fertilizer.

Although the $NaHCO_3$ treatment did not inhibit the growth of Rhodes grass, the concentration of phosphorus in the tops was markedly reduced by it. If the deleterious effect of the $NaHCO_3$ treatment on Dallis grass is to be explained through its effect on the phosphorus content of the tops, it will be necessary to postulate that phosphorus metabolism is markedly different in the two species. This has not been demonstrated. The plants illustrated in figure 3 were assayed for phosphorus, and the concentrations were found to be the same for plants grown at pH 6 and pH 8 base nutrient, suggesting that the depressing action of the $NaHCO_3$ treatment on phosphorus content cannot be explained on the basis of alkalinity alone. ARNON and JOHNSON (**1**) observed that pH 8 had very little effect on phosphate absorption by several species of plants.

STEWARD and PRESTON (**31**) have reported that the bicarbonate and carbonate anions have a unique effect on respiration and metabolism. These authors stated that "this is the only case yet encountered . . . in which an increased concentration of potassium salt decreased respiration and metabolism of potato discs. It is evident that this effect is due specifically to HCO_3 and H_2CO_3."

It is eminently possible that the intense chlorosis and very poor growth of Dallis grass on the $NaHCO_3$ cultures was directly related to disturbances in phosphorus and iron metabolism. OLSEN (**25**) ascribed the chlorosis of corn plants grown on nutrient cultures to the precipitation of ferric phosphate in the conductive tissues with the resultant unavailability of iron to leaf parenchyma cells. BIDDULPH (**2**) grew bean plants in nutrient solutions that induced varying degrees of chlorosis and added either radioactive iron or radioactive phosphorus to the solutions. Radioautographs of the various tissues showed very clearly that on cultural treatments that induced chlorosis, iron and phosphorus accumulated intensively in the roots and conductive tissues with relatively low levels in the leaf parenchyma. That is, the distribution of these two elements differed markedly in chlorotic and green plants. BIDDULPH (**2**) pointed out that the localization of iron and phosphorus in the chlorotic plants was probably due to precipitation as ferric phosphate. The depressive effect of $NaHCO_3$ on growth of Dallis grass may well have taken place by such means.

Summary and conclusions

1. Dallis grass (*Paspalum dilatatum* Poir.) and Rhodes grass (*Chloris gayana* Kunth.) in sand culture were subjected to seven series of treatments: In addition to the base nutrient (control) solution,

six salts were added singly to base nutrient solution—$NaHCO_3$, $MgCl_2$, NaCl, $NaNO_3$, Na_2SO_4, and $CaCl_2$. The first of these salts was added at 12 m.e./l., the second to the extent of 1 atmosphere osmotic pressure, and each of the others at 2 atm. OP.

2. On a relative basis, Dallis grass is much less salt tolerant than Rhodes grass. In addition to its greater salt tolerance, Rhodes grass is also the higher producer of forage on an absolute basis.

3. Although the $NaHCO_3$ treatment was without effect on the growth of Rhodes grass, it was exceedingly toxic to Dallis grass. The difference in response of these two grasses to $NaHCO_3$ was the most striking observation during the experiment.

4. Rhodes grass accumulated sodium readily in the tops, whereas the sodium content of Dallis grass was characteristically low and was increased only slightly even by a high concentration of sodium in the substrate.

5. The $NaHCO_3$ treatment was associated with a lowering of the sulfate and phosphate content of both grasses, but the effect could not be entirely ascribed to high pH. It is suggested that the severe chlorosis and depressed growth of Dallis grass on the $NaHCO_3$ cultures may have resulted from disturbances in phosphorus and iron metabolism.

6. The different treatments brought about marked variations in the mineral composition of both grasses. With the exception that the poor growth of the $MgCl_2$ cultures was directly related to excessive accumulations of magnesium, the observed growth responses could not be conclusively associated with the status of accumulated inorganic ions.

7. Excepting the $MgCl_2$ and $NaHCO_3$ treatments which induced specific toxic symptoms, growth response to the salinized culture solutions was most logically interpreted in terms of physiological availability of water.

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SOME EFFECTS OF MALEIC HYDRAZIDE ON PLANTS

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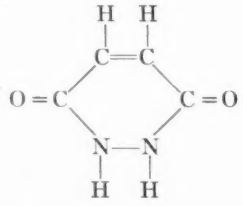
Introduction

In the field of plant growth-regulators, considerable interest has developed in the use of maleic hydrazide.[2] It has the structure



and is commonly employed as the diethanolamine salt. A wide variety of responses to it has been noted, depending upon species, age of plant, and concentration of chemical employed. It was reported to have a pronounced but temporary inhibiting effect on tomatoes (**18**) and a delaying action on blossoming of certain fruits (**22**) and also was found to have a slow herbicidal effect, particularly on grasses (**6**). Dicotyledonous plants were relatively resistant, although here, as for grasses, age was a critical factor; younger plants were more susceptible (**5**).

This paper reports studies designed to further understanding of the mechanism by which plants respond to maleic hydrazide.

Experimentation

Greenhouse experiments with barley and mustard

Mustard (*Brassica arvensis* L.) seeds were planted in gallon cans containing silt loam soil, and 2 days later barley (var. Sacramento) was planted between the mustard. The plants were thinned to ten of each species per can, and when 14 and 12 days old, respectively, they were sprayed to runoff (about 15 ml. per can) with maleic hydrazide (MH; diethanolamine formulation) and 2,4-D (a 1:1 ethyl ethanolamine–ethyl diethanolamine formulation of 2,4-dichlorophenoxyacetic acid),[3] both alone and in combination. Vatsol[4] (0.05%) was added as a spreader to all solutions. At the time of spraying the barley was about 10 inches tall, in the third leaf stage; the mustard was about 2 inches tall, in the second leaf stage. Five treatments were given, with five cans per treatment, each can containing ten barley and ten mustard plants: 0.1% 2,4-D; 0.2% MH; 0.4% MH; 0.1% 2,4-D + 0.2% MH; and controls.

Effect of 2,4-D.—The mustard was killed in a few days by the 2,4-D spray. The barley was retarded in growth slightly and became noticeably darker green than the controls but otherwise was apparently not affected.

Effect of maleic hydrazide.—After the first week barley was retarded but showed no other evident injury. During the second week leaves appeared mottled and, relative to the untreated plants, had become noticeably thicker and more brittle, the latter evidenced by snapping when a leaf was bent double. Anthocyanin pigments were not observed to any extent, in contrast to the response to

[1] Present address: Citrus Experiment Station, Riverside, California.

[2] Supplied by the Naugatuck Chemical Division, United States Rubber Co., Naugatuck, Connecticut.

[3] Sharsol 161, Sharples Chemicals, Inc., Philadelphia.

[4] American Cyanamid Co.

this chemical by many other plants. Sticky drops of exudate appeared on leaf surfaces, at first colorless, later turning light amber. NAYLOR and DAVIS (**14**) reported such exudate in their work with corn. These drops were not localized but appeared any place on either surface. Often a portion of the tissue beneath the drops was necrotic and brownish. An analysis of the exudate is reported in a later section. Five weeks after spraying all the barley was dead.

The mustard plants were severely retarded in growth and exhibited some burning of leaf margins and anthocyanin coloring in the first stages of injury. Although many plants eventually died, some recovered and flowered and fruited normally. In contrast to an age at flowering of about 4 weeks for the controls, however, the treated plants required 8 weeks before flowering.

Doubling of the MH concentration from 0.2 to 0.4% was reflected in slightly greater injury and somewhat more rapid killing of barley, but the effect was not pronounced.

The selective and opposite effects of 2,4-D and MH on barley and mustard are thus demonstrated. Figure 1 indicates the appearance of plants 26 days after spraying.

EFFECT OF COMBINATION OF 2,4-D AND MH.—A clear-cut antagonism between these two substances developed. Mustard apparently was killed as rapidly but barley clearly less rapidly than with MH alone at the same concentration. Four weeks after spraying, plants treated with 0.2% MH were practically devoid of green pigments, while the plants receiving the combination spray (0.2% MH + 0.1% 2,4-D) showed considerable green color (fig. 2). A possible explanation is that 2,4-D stimulates depletion of carbohydrate reserves (**16, 19**), whereas maleic hydrazide causes accumulation, and that injury resulting from accumulation might be delayed when 2,4-D is present. Time series of analyses for both maleic hydrazide alone and in combination with 2,4-D, running throughout the life of the plants, are needed.

FIGS. 1, 2.—Barley and mustard plants. Fig. 1 (*above*), control plants (*left*) compared with plants sprayed with 0.1% 2,4-D (*center*) and with 0.2% maleic hydrazide (*right*). Fifteen days after treatment. Stunted but living mustard plants in right-hand can are hidden by barley. Fig. 2 (*below*), antagonism of 2,4-D and maleic hydrazide. *Left to right:* control plants, sprayed with 0.2% maleic hydrazide, sprayed with 0.2% maleic hydrazide + 0.1% 2,4-D.

EFFECT OF MALEIC HYDRAZIDE ON CARBOHYDRATE METABOLISM OF BARLEY

There are several indications that leaves sprayed with MH accumulate sugars. In corn leaves so treated NAYLOR and DAVIS (**14**) found large increases of sucrose and reported drops of viscous exudate containing sucrose on leaf sur-

faces. Anthocyanin pigmentation is an additional general response and is pronounced in some dicotyledonous species, such as *Rumex crispus* and *Erodium cicutarium*, and develops also to a lesser extent in certain grasses (**5, 14**). Such pigmentation is believed to indicate high sugar content.

treatments: (*a*) sprayed with 0.2% MH, (*b*) sprayed with 0.4% MH, and (*c*) controls.

EXTRACTION OF DRY MATERIAL.—Harvested shoots were weighed and immediately dried *in vacuo* at 70° C. to constant weight and subsequently ground to 20 mesh (per sq. in.) in a Wiley

TABLE 1

DATA ON BARLEY SHOOTS TREATED WITH MALEIC HYDRAZIDE (MH)

	0.2% MH		0.4% MH		CONTROLS	
	a	*b*	*a*	*b*	*a*	*b*
	Total weight					
Fresh weight, gm.*	17.2	20.0	16.0	16.0	25.4	22.1
Dry weight, gm.*	4.65	5.52	4.05	4.52	3.95	3.62
Dry weight as % of fresh weight	27.0	27.6	25.3	28.2	15.6	16.4
	Percentage of dry weight extractable					
By 80% ethanol	39.2	46.0	40.4	44.0	39.2	36.8
By water	29.6	23.2	25.2	24.8	10.8	11.6
Total extracted	68.8	69.2	65.6	68.8	50.0	48.4
	Nitrogen content, percentage of dry weight					
N content	1.97	1.89	1.89	1.80	1.70	1.67

* Each figure represents ten shoots.

Samples of barley were collected from the series of barley and mustard cultures described above. These plants were started from seed on April 28, sprayed on May 10, and harvested on May 25 by removing the entire shoot $\frac{1}{4}$ inch above the soil. Treated plants were still living though clearly injured; their leaves were mottled, yellowish green, and retarded in growth. The treated leaves measured only 37 cm. high, leaves of control plants 45 cm.

Two cans each containing ten plants were used as duplicates in each of three

mill. Plants treated with MH were lower in fresh weight but higher in dry weight than the controls (table 1).

Samples of 2.5 gm. each were extracted for 6 hours with 80% ethanol in a Soxhlet apparatus. The residues were dried and reweighed and then mechanically shaken with 200 ml. of water for 4 hours at 25° C. The residues after filtering and drying were again weighed. Table 1 gives data from these extractions.

The variation among duplicates in the alcoholic extraction may be explained by

the fact that barley fructosans are not completely insoluble in 80% alcohol (1), together with a varying efficiency of the different extractors. This is borne out by the results with aqueous extraction, and is reflected in the sugar analyses (table 2).

The treated plants were thus higher in soluble material and lower in insoluble substance than the controls. That the difference may be due to less cell-wall material in the treated plants is indicated by total (Kjeldahl) nitrogen[5] analyses using unextracted dry samples (table 1).

SUGAR DETERMINATIONS.—The method for reducing sugar employed ceric oxidimetry and followed the procedure of HASSID (10) with the exception that charcoal and talc were omitted from the clearing procedure. Neutral lead acetate was used to clear, and the excess of lead was removed by Na_2HPO_4.

In addition to reducing sugar analysis, the material was hydrolyzed in two ways: (*a*) by 0.02% Wallerstein's invertase, 2 hours at 29° C., and (*b*) by adding 10 ml. of 1:1 HCl to 50 ml. of plant extract and then incubating for 24 hours at 29° C.

The invertase value minus reducing sugar is considered to be a measure of sucrose content. This interpretation is in question with respect to the aqueous extracts, since all the sucrose should have been extracted by the 80% ethanol. It is more likely that the values obtained are the result of invertase action on fructosans. Since fructosans are known to be normal constituents of barley (3), and since they are only slightly susceptible to invertase but are hydrolyzed rapidly by acids (2), acid-hydrolysis values include both sucrose and fructosans. A measure of fructosan content is given by the difference between the acid value and the invertase value. Actually the fructosan values are probably low by the amount hydrolyzed by invertase under the conditions of the experiment. This amount varied from 4% to 20% in the work of ARCHBOLD and BARTER (3), using a rather pure fructosan preparation isolated from barley. In the present tests, assuming that all the sucrose value in the aqueous extracts represents the action of invertase on fructosans, lower values were obtained, about 1% for the treated plants and 2–4% for the controls.

Tables 2 and 3 show data obtained in sugar analyses.

Additional evidence that the difference between acid and invertase values is attributable to fructosans was given by the use of MCRARY and SLATTERY's (12) procedure for fructosans as used in guayule analysis. This indicated a high fructose content and was convincing (table 4).

ANALYSIS OF EXUDATE.—Fifteen days after barley plants were sprayed with maleic hydrazide, 0.255 gm. of the exudate was collected from 0.2%-treated plants. This was dried, dissolved (readily) in cold water, and gave a clear solution. Sugar and total nitrogen analyses were carried out as above (table 5). An additional 0.29-gm. sample was collected 25 days after spraying from plants which at this time were quite yellow, and this was carried through the sugar analyses.

The data indicate that there was no fructosan in the exudate and that the predominant constituent was sucrose. The nitrogen values were very close to those obtained for the whole shoot. It is possible that this may be largely phloem exudate.

There is no ready explanation for the

[5] Kindly determined by Dr. K. L. OLSEN, using a micro-Kjeldahl apparatus and a method in which the ammonia is collected in boric acid (15).

invertase values exceeding the acid values for the first exudate sample. Clearing with lead lowered the reducing sugar value from an average of 14.2% to 12.2%, although there was hardly any precipitate upon adding lead acetate to the solution.

Summarizing the results of this section, the increase in dry weight displayed by barley plants sprayed with 0.2% and

TABLE 2

ANALYSIS OF BARLEY EXTRACTS, SUGAR CONTENT AS GLUCOSE PERCENTAGE OF DRY WEIGHT

	0.2% MH		0.4% MH		CONTROLS	
	a	*b*	*a*	*b*	*a*	*b*
	Alcoholic extract					
Unhydrolyzed	6.4	6.4	6.7	6.8	6.7	6.9
After acid hydrolysis	29.7	35.0	28.2	34.5	18.7	19.9
After invertase hydrolysis	9.5	9.5	9.5	9.9	9.8	9.8
	Aqueous extract					
Unhydrolyzed	0.40	0.26	0.26	0.37	0.19	0.21
After acid hydrolysis	23.1	22.1	19.6	19.0	2.7	4.4
After invertase hydrolysis	0.63	0.43	0.46	0.57	0.29	0.31

TABLE 3

SUGAR DATA OF TABLE 2 IN TERMS OF REDUCING SUGAR, SUCROSE,* AND FRUCTOSAN.† PERCENTAGE OF DRY WEIGHT

	0.2% MH		0.4% MH		CONTROLS	
	a	*b*	*a*	*b*	*a*	*b*
	Alcoholic extract					
Reducing sugar	6.4	6.4	6.7	6.8	6.7	6.9
Sucrose	2.9	2.9	2.7	2.9	2.9	2.8
Fructosan	18.4	22.9	16.8	22.2	8.0	9.1
	Aqueous extract					
Reducing sugar	0.40	0.26	0.26	0.37	0.19	0.21
Sucrose (?)	0.22	0.13	0.14	0.14	0.09	0.09
Fructosan	20.2	19.5	17.2	16.6	2.15	3.66

* Sucrose = (invertase value − reducing sugar) × 0.95.

† Fructosan = (acid value − invertase value) × 0.90.

0.4% maleic hydrazide was due almost entirely to an accumulation of fructosan polysaccharides. Very little change occurred in the concentration of reducing sugars or sucrose. The drops of sticky exudate appearing on the leaves of treated plants were high in sucrose, with fructosans absent if interpretations are correct. There is thus the problem of explaining the change in carbohydrate metabolism responsible for a high fructosan content of the leaf tissue, on the one hand, and a high sucrose content of the exudate, on the other. If this excretion originated in the phloem, there arises the possibility that enzymatic mechanisms exist which can convert fructosan to sucrose within the phloem.

TABLE 4

FRUCTOSE CONTENT* OF BARLEY EXTRACTS ANALYZED BY COLORIMETRIC RESORCINOL METHOD (12). PERCENTAGE OF DRY WEIGHT

	0.2% MH		0.4% MH		CONTROLS	
	a	*b*	*a*	*b*	*a*	*b*
Alcoholic extracts...	20.4	25.2	18.8	24.8	7.2	8.4
Aqueous extracts...	21.0	21.4	16.2	18.0	1.6	2.6

* Includes that combined as sucrose or fructosan.

TABLE 5

ANALYSIS OF EXUDATE COLLECTED FROM BARLEY LEAVES 15 AND 25 DAYS AFTER SPRAYING WITH MALEIC HYDRAZIDE. PERCENTAGE OF DRY WEIGHT

	15 DAYS			25 DAYS		
	a	*b*	Av.	*a*	*b*	Av.
Reducing sugar	6.4	6.8	6.6	12.1	12.3	12.2
After acid hydrolysis	66.2	65.3	65.8	81.5	83.5	82.5
After invertase hydrolysis	77.6	78.9	78.3	83.2	83.2	83.2
Total nitrogen	1.93	1.98	1.95	.....	.....	.....

EFFECT OF MALEIC HYDRAZIDE ON CURVATURE REACTION OF BEAN PLANTS

The accumulation of sugar in leaves of plants treated with maleic hydrazide might be the result of a failure in translocation. Since 2,4-D is known to be translocated along with the food products elaborated by photosynthesis (**11, 13, 17, 20, 21**), this substance can be used as a general indicator of translocation (**7**).

The following experiments were performed to determine whether maleic hydrazide had a specific effect upon translocation. Red Kidney beans were grown in individual 4-inch pots to a stage where the primary leaves were almost fully expanded but the terminal bud had not developed appreciably above the second node. A solution of 0.2% maleic hydrazide was painted with a soft brush on one primary leaf of each of eight such plants. After 22 hours 5 micrograms of 2,4-D dissolved in 10 microliters of 2% ethanol were applied by means of a micropipette to the upper surface of each of the primary leaves. Curvature in a direction

away from the leaf treated with maleic hydrazide could be evidence of increased translocation from that leaf; whereas curvature in the opposite direction might indicate more rapid translocation from the control leaf. A maximum curvature of 73°.5 toward the treated leaf was observed 13 hours after application of the 2,4-D. Although it could be concluded from these results that maleic hydrazide delayed or prevented the translocation of 2,4-D and possibly other substances, there remains the possibility that the maleic hydrazide could itself have been translocated into the stem where it counteracted the 2,4-D response. To clarify further this response, the following experiment was performed:

Three groups of ten bean plants were treated by painting 0.2% MH solution on one primary leaf. After 48 hours a drop of solution containing 5 micrograms of 2,4-D was applied to each primary leaf (treatment A), to the epicotyl on the side of the treated leaf (treatment B), or to the opposite side of the epicotyl (treatment C). Curvature values for the epicotyls for various times up to 24 hours after application of the 2,4-D are given in table 6. It is shown (treatments B and C) that maleic hydrazide or some secondary effect resulting from it was transported from the leaf into the epicotyl. Although translocation of 2,4-D from the MH-treated leaf seemed to be retarded (treatment A), the data do not indicate whether the difference in curvature was due to an effect of MH on curvature or upon translocation of 2,4-D. Several preliminary tests proved that MH has no direct growth effect on bean seedlings resulting in curvature. The most plausible conclusion seems to be that in some way it hinders 2,4-D movement from the leaf to the epicotyl.

TABLE 6

EFFECT OF MALEIC HYDRAZIDE UPON CURVATURE RESPONSE (DEGREES) OF BEAN SEEDLINGS TO 2,4-D*

TREATMENT, 5 MICROGRAMS 2,4-D	HOURS AFTER APPLICATION OF 2,4-D						
	2	3	4	5	6	11	22
A (on both leaves)	0	3.3 ±1.1†	6.8 ±1.8	10.4 ±2.9	17.1 ±4.5	39.3 ±10.5	39.0 ±9.9
B (on epicotyl on side of treated leaf)	2.0 ±1.8	7.5 ±1.3	8.7 ±1.2	11.0 ±2.6	11.7 ±2.8	8.9 ±5.8	6.5 ±5.8
C (on epicotyl on side opposite treated leaf)	9.0 ±1.8	21.2 ±4.3	30.1 ±5.9	38.9 ±4.9	46.5 ±4.4	54.3 ±7.4	54.5 ±6.7

* Group A curved in response to the leaf that had not been treated with MH. Groups B and C curved away from the 2,4-D–treated side of the epicotyl.

† The ± figures are standard errors of the mean calculated by the usual method for small samples (**8**).

Discussion

Several clues to the mode of action of MH are available. Cell division ceases soon after treatment (**9**); symptoms similar to those of curly-top and shoestring viruses appear on certain species (**5**); grasses seem to be completely inhibited, and carbohydrates apparently accumulate in the foliar parts of the plants.

The effect of MH on cell division seems to be direct and specific. The

severity of the reaction depends on the concentration applied. All plants recover from low dosages, but dicotyledonous plants seem able to tolerate more than do grasses.

Development of symptoms resembling those of virus infection and the accumulation of carbohydrates in leaves are more complex responses. Since curly-top causes phloem necrosis and sieve-cell obliteration, it is possible that these responses might be causally related and attributable to disturbance of the phloem tissues. GIROLAMI's work (9) seems to confirm this conclusion. However, it remains to be demonstrated whether MH acts directly on the phloem or whether the effect is a secondary one.

If maleic hydrazide directly affects the phloem, advanced callusing of the sieve plates might be found within 24–48 hours. Bean plants were treated with maleic hydrazide as above, and sections of midrib and petiole of the treated primary leaf were killed in IKI and counterstained with aniline blue. In tests made 1, 7, and 25 days after treatment, no instance of abnormal callus formation was observed. The technique again gave no evidence in this direction when repeated, using squash petiole and stem, and midrib of barley leaves in various stages of injury.

Tissues of treated bean plants, however, became progressively more filled with starch. Material examined after 25 days appeared gorged with starch grains, including accumulations in collenchyma and epidermis. Sections of stem showed an abundance of starch in pith and cortex at all levels.

As a gross response to painting one primary bean leaf with MH, it was found that the opposite leaf also accumulated starch. Thus the effect, whether actual translocation of MH or of something else, seemed to be transmitted to the other leaf. Removal of the treated leaf resulted in growth of axillary buds, but with the plant intact no such initiation occurred, at least not within 25 days. No apical growth occurred in any plant.

GIROLAMI (9) observed that flax treated with MH displayed phloem necrosis near the stem apex which could account for loss of apical dominance.

Within a few hours after treatment MH apparently migrates into the phloem, where it moves at least for short distances. In common with many other materials such movement might be expected to accompany that of foods (4). It undoubtedly results in more widespread inhibition of cell division and is responsible for the general cessation of growth.

As MH moves in the phloem, it apparently affects sieve-tube differentiation (9) and finally causes necrosis of this conducting tissue. The relation of this to the observed accumulation of fructosan in barley and of starch in a number of species is not clear. There appears to be no unusual callusing of sieve tubes such as would be expected if direct injury resulted from presence of the MH.

Summary

1. Young barley plants sprayed with maleic hydrazide (MH) showed a smaller fresh weight but greater dry weight than untreated plants after 12 days.

2. The increase in dry weight was due predominantly to accumulation of fructosan polysaccharides; sugars remained the same.

3. Drops of exudate on treated leaves contained mostly sucrose, no fructosan, and showed total nitrogen equal to that of the plant material.

4. 2,4-D (2,4-dichlorophenoxyacetic acid) partially antagonized the effect of maleic hydrazide on barley plants.

5. Maleic hydrazide interferes with translocation and the curvature effect of 2,4-D in the epicotyl of young bean plants. Either MH itself is translocated, or some secondary effect is transmitted from the leaf to the epicotyl.

6. No evidence was obtained of abnormal callus formation on sieve plates as an effect of maleic hydrazide.

7. In bean plants, starch accumulation is a visible response to MH treatment.

8. Possible mechanisms of action of MH are discussed.

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RESPONSE OF FIBROUS ROOTS OF SUNFLOWER AND TOBACCO TISSUE CULTURES TO PLANT GROWTH SUBSTANCES[1]

MICHAEL LEVINE

Introduction

The cultivation of segments of fleshy roots under aseptic conditions on synthetic media has been studied principally by GAUTHERET (**2, 3**). His studies were focused primarily on the nutritional requirements of these tissues and indicated that segments of certain roots like the carrot, chicory, and *Scorzonera* needed the mineral salts and sucrose furnished by Knop's solution, the oligodynamic elements suggested by BERTHELOT, and, in addition, the growth substance indoleacetic acid (IAA). These segments produced roots in culture and, in the case of chicory, when deprived of IAA, produced buds.

The problem of prolonged and continuous cultivation *in vitro* of such structures as excised roots was solved by WHITE (**14**) and later by others. NOBÉCOURT (**9, 10**) observed that some fibrous roots from old tissue cultures of the carrot gave rise to tissue masses and to cauline structures.

A recent study (**7**) showed that excised fibrous roots of carrot tissue cultures, when planted on standard media, grew in length and branched. Their growth seemed unaffected by the addition of coconut milk. When planted on a medium containing IAA (5 mg/l), however, linear growth was impaired, and nodules of hyaline, translucent tissue were formed along the upper surface of the root. New white roots appearing from these nodular masses grew rapidly and penetrated the agar medium. When these masses were separated by cutting the root and transplanted to a fresh medium lacking IAA, they produced shoots and leaves giving evidence of complete differentiation. When the medium included both IAA and coconut milk, the response differed. Along the upper length of the seeded root, opaque, pigmented, nodular masses developed. These were generally green in color with some red or colorless portions. These nodules represented the now well-known callus tissue masses described by GAUTHERET and NOBÉCOURT and were like those that appeared in carrot tissue cultures in this laboratory. These masses when separated and transplanted were more resistant to differentiation, yet cauline and foliar elements were formed in subsequent transplant generations.

These observations stimulated the study of fibrous roots from additional tissue cultures: sunflower (*Helianthus annuus* var. Russian) and several forms of tobacco. Sunflower cultures obtained from stem tissue produced an abundance of roots on IAA media, but so far they have yielded no stems or leaves. Tissue cultures of *Nicotiana affinis* derived from stem segments yielded many roots, with leafy stems occurring frequently (**6**). Cultures of the stem segments of a tobacco hybrid (*N. glauca* × *N. langsdorffii*) and of the leafy "tumors" that develop spontaneously on the adult hybrid also produced many roots. Tissue cultures of the hybrid grew abundantly

[1] Supported by a grant-in-aid, American Cancer Society, Inc., upon the recommendation of the Committee on Growth, National Research Council.

but differentiated more sparingly than those of *N. langsdorffii* which behave like those of *N. affinis*. *N. glauca* tissue has grown poorly *in vitro*.

Material and methods

Tissue culture masses of *Helianthus annuus* var. Russian, *Nicotiana affinis*, and a hybrid (*N. glauca* × *N. langsdorffii*) were used to furnish fibrous roots. The fibrous roots were grown on standard media such as compounded by WHITE (14) and HILDEBRANDT *et al.* (4) for tobacco and sunflower; also on Knop's medium to which BERTHELOT's oligodynamic substances were added (2). To these solutions made with anti-pyrogen water was added 0.1 mg/l or 5 mg/l IAA or one of three chemical carcinogens (10mg/l)—1,2,5,6-dibenzanthracene, 3,4-benzpyrene, or 20-methylcholanthrene—or autoclaved coconut milk (10%), or a combination of coconut milk with IAA. Shredded agar (6.5–7.5 gm/l) washed for 3 days was added to this medium. The following abbreviations are used to refer to the various compounds and media: B = 3,4-benzpyrene; D = 1,2,5,6-dibenzanthracene; M = 20-methylcholanthrene; CM = coconut milk; IAA = indoleacetic acid; Ws—WHITE's medium; Wsf = HILDEBRANDT *et al.* medium for sunflower; Wt = HILDEBRANDT *et al.* medium for tobacco.

The media were distributed in 125-ml. Erlenmeyer flasks in 25-ml. amounts, and the fibrous roots of known origin were aseptically planted on the surface. The roots selected measured approximately 4 cm. long and were near 1 mm. in diameter. Excised roots were seeded from known cultures in series, so that a large number of root cultures, forty to fifty, were grown on standard media and an equal number on IAA, CM, CM plus IAA, D, B, or M. Division of the treated roots was required at 6–8 weeks; each part was tested on basic medium and then transplanted to other substances in an attempt to bring about differentiation of shoots. Frequently two excised roots or roots with multiple branches were used in one culture flask. The medium remained moist and suitable for as long as 3 months. Series of fibrous root cultures derived from the same source but under different treatment were compared with controls grown on basic medium only. Photographic records of the cultures were made, and many cultures were prepared for microscopic study.

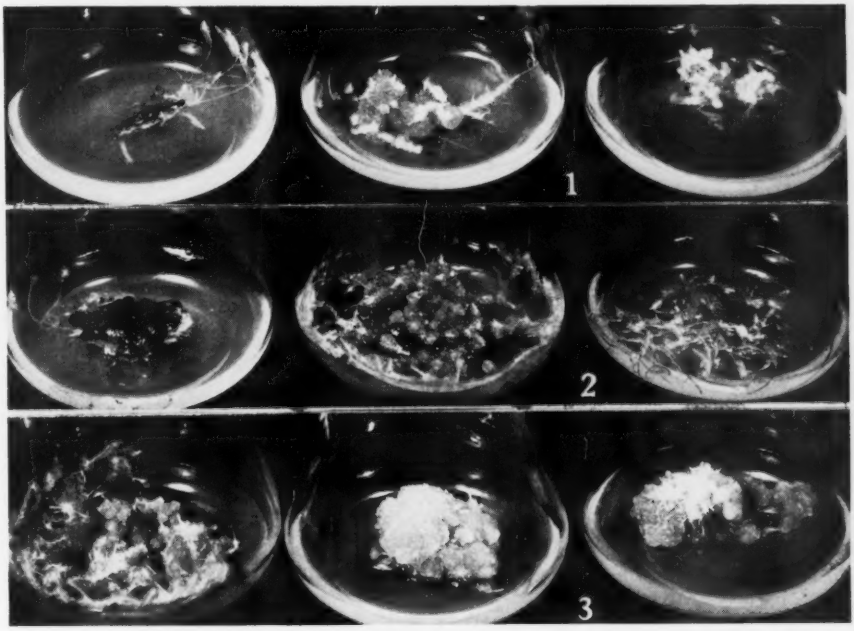
Observations

SUNFLOWER

When excised fibrous roots, which had been produced by tissue cultures of the sunflower grown on any of the media alone or with added IAA, D, B, or M, were seeded on a standard medium, they grew in length and frequently produced a number of branches. Terminal parts of these roots became covered with an abundance of root hairs when they emerged from the agar. Roots planted on a medium with coconut milk behaved like those grown on a basic medium or grew little, and their branches were small. Occasionally small, barely perceptible nodules appeared close together on the seeded root.

Cultures of fibrous roots with IAA or IAA and CM produced little or no linear growth but developed granular jelly-like masses along the length of the excised root. These light-brown masses gave a beaded appearance to the growth, but, as they aged, they fused and became dark brown. New roots appeared from their under and upper surfaces. Those from the upper surfaces were exposed to

the humid air in the flask, and their distal portions became covered with a dense outgrowth of root hairs. Growth on the lower concentration of IAA (0.1 mg/l) produced small calluses about the bases of the secondary roots that developed at the same time.



FIGS. 1–3.—Excised fibrous roots from sunflower tissue culture. ×4/9. Fig. 1, grown on (*left*) standard medium (Wsf) and on (*center, right*) medium with indoleacetic acid (IAA). Roots in left two cultures came from standard medium; root in right flask was derived from methylcholanthrene culture (M). Fig. 2, calluses on fibrous root cultures grown on (*left*) Wsf, (*center*) Wsf with coconut milk (CM), and (*right*) Wsf with IAA. Fig. 3, calluses on fibrous roots grown on (*left*) Wsf with dibenzanthracene (D), (*center*) Wsf with benzpyrene (B), and (*right*) Wsf M.

Figure 1 illustrates three cultures of a series in which the left culture shows a root with several branches grown on standard medium. The original transplanted root appears as a dark strand in the photograph, while the new growths are thin, fibrous, and white, and the aerial portions are covered by root hairs. The center culture was seeded on Wsf with IAA (5 mg/l) and was derived, as was that in the flask on the left, from a growth on standard medium. The confluent gelatinous masses became covered by short roots with root hairs, while long, glistening white roots appeared from the underside of the tissue mass. The fibrous root that had been planted in the right-hand culture on Wsf with IAA was excised from a tissue mass grown on Wsf with M (10 mg/l) for five previous transplant generations. The excised root became covered with a similar type of gelatinous mass seen in the center flask, but many short roots developed from its surface. These results indicate that the medium upon which the roots are seeded determines the nature of the reaction.

Clones of sunflower tissue cultures that had been started in 1946 from stem segments yielded fibrous roots that were transferred to Wsf IAA on August 17, 1948, and subcultured on October 4, 1948, on similar medium. The resulting granular, hyaline tissue masses were divided and seeded on June 20, 1949, on media as shown in figure 2: (*left to right*) Wsf, Wsf CM (10%), Wsf IAA (5 mg/l) and in figure 3: Wsf D, Wsf B, Wsf M (D, B, and M all at 10 mg/l). The tissue masses thus developed resembled those derived from subcultures of the sunflower stem segments. The carcinogen-treated cultures (fig. 3) produced massive translucent growths with few roots.

Differentiation of these tissue masses of the sunflower to form cauline or foliar elements has not been observed. It is evident that such masses may be started from a stem segment or from a fibrous root. As NOBÉCOURT (**9, 10**) pointed out, the tissue mass may give rise to an organ, and the organ may give rise to a tissue mass. This is based on the assumption that the tissue culture is composed of heterogeneous cellular elements. Homogeneous parenchymatous cultures described by MOREL (**8**) for the grape, or the culture of the bacteria-free crown-gall tissue with a sparse scattering of lignified vessels among the predominantly parenchymatous cells that compose it, could be expected to form organs; yet in the latter case the possibility of producing roots would be better than in the grape tissue cultures where only parenchyma cells are found. It appears that a homogeneous parenchymatous tissue mass is incapable of further differentiation. Crown gall is not a homogeneous tissue. The fortuitous isolation of parenchyma cells from a bacteria-free crown gall grown *in vitro* does not appear to represent the complete morphology of the crown-gall tumor.

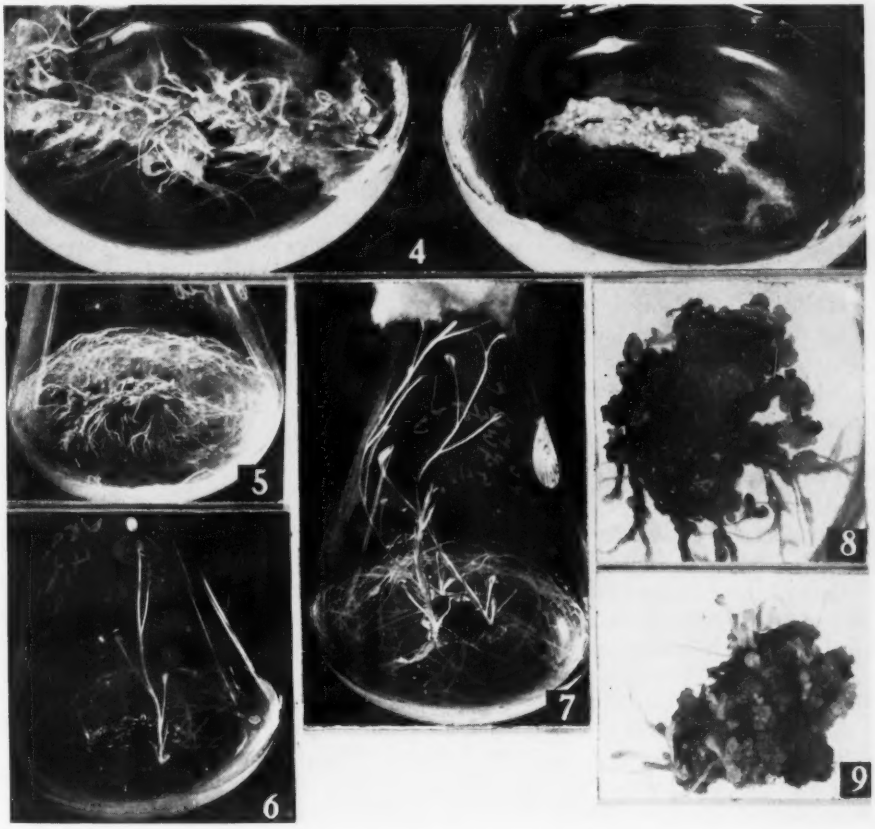
TOBACCO

N. AFFINIS.—Fibrous roots from cultures of *N. affinis* responded to IAA very much like those of the carrot (**7**). When Knop's solution was used as the basic medium and IAA was added, however, the difference in reaction was of interest. Fibrous roots were planted on this medium with 5 mg/l IAA (fig. 4, right) and on Wsf with 0.1 mg/l IAA (left). Both cultures are from a series of twenty set out on July 25, 1949, and were photographed on September 1, 1949. Small hyaline masses appeared on the root of the left-hand culture within a week, followed shortly thereafter by a rapid growth of new roots. The original explant failed to increase in length, but from it developed a small plantlet with three to four leaves. The culture on the right (fig. 4) shows the root covered by numerous small nodules with few or no new roots. This response might reflect on the inadequate nutrient supply in Knop's solution; concentrations of IAA (5 mg/l) generally inhibit bud formation.

Fibrous roots with their hyaline caluses grown on IAA with Wt or Wsf for 5–6 weeks were cut into small pieces, each including a small tissue mass. These were planted on basic media to which CM, CM with IAA (5 mg/l), D, B, or M had been added. The root cultures were divided and seeded on December 5, 1948, and photographed 3 months later. Parts of the cultures transferred to IAA are shown in figure 5. The upper surface of the hyaline translucent mass was covered by short roots, while long roots with many branches penetrated the agar from its lower surface. No further differentiation was observed. Another part of the fibrous root transplanted to basic me-

dium (fig. 6) showed the formation of a well-formed plantlet from a small, brownish tissue mass. The agar was penetrated by long and branching roots. The part of the IAA fibrous-root cultures transferred to D in Wsf medium produced an abundance of shoots from a relatively small tissue mass. On March 16, 1949, the culture shown in figure 7 had developed rosettes of leaves, and a fortnight later the shoots expanded. In this culture the evidence appears clear that some buds of this tobacco may develop on fibrous roots. The CM, CM plus IAA, B, and M cultures developed large tissue masses, and the last two types of cultures, B and M, produced an abundance of roots. Complete differentiation in the latter was not uncommon.

Indoleacetic acid and alpha-naphthal-



FIGS. 4–9.—*Nicotiana affinis*. Figs. 4, 8, 9, ×2/3; figs. 5–7, ×4/9. Fig. 4, fibrous roots from tissue culture, grown on (*left*) Wsf IAA (0.1 mg/l) and (*right*) Knop's medium with IAA (5 mg/l). Fig. 5, callus on fibrous root grown on Wsf IAA (5 mg/l). Fig. 6, callus grown on Wsf, with differentiated plantlet. Fig. 7, callus grown on Wsf D (10 mg/l). Fig. 8, callus of fibrous root developed on Wt IAA (0.1 mg/l), transferred to Wt CM on June 21, 1949; produced a few well-developed plantlets; photographed August 17, 1949. Fig. 9, callus grown on Wsf CM IAA (5 mg/l), transferred to Wsf CM on May 4, 1949; many plantlets arose from nodular tissue mass; photographed August 17, 1949.

eneacetic acid have been studied principally for their rhizogenous and other growth-promoting properties. SKOOG in 1944 (11) and more recently in collaboration with CHENG TSUI (12) claimed an inhibitory effect of those substances on cauline and foliar differentiation. The writer's observations (5, 6) on carrot and tobacco tissue cultures seem to substantiate these findings. The concentration of IAA used and the time of its use after preparation, however, seem to influence the results significantly. Comparatively fresh cultures with IAA (0.1 mg/l) and old cultures with IAA (5 mg/l) have produced typical carrot and tobacco plantlets. In the latter case the writer has suggested that the decomposition and utilization of the IAA may be responsible for the loss of the caulineinhibiting effect. Use of media made with solutions of IAA (5 mg/l) that had been prepared 60 days previously, however, resulted in the development of hyaline calluses on fibrous roots and satisfied the needs of carrot and sunflower tissue cultures.

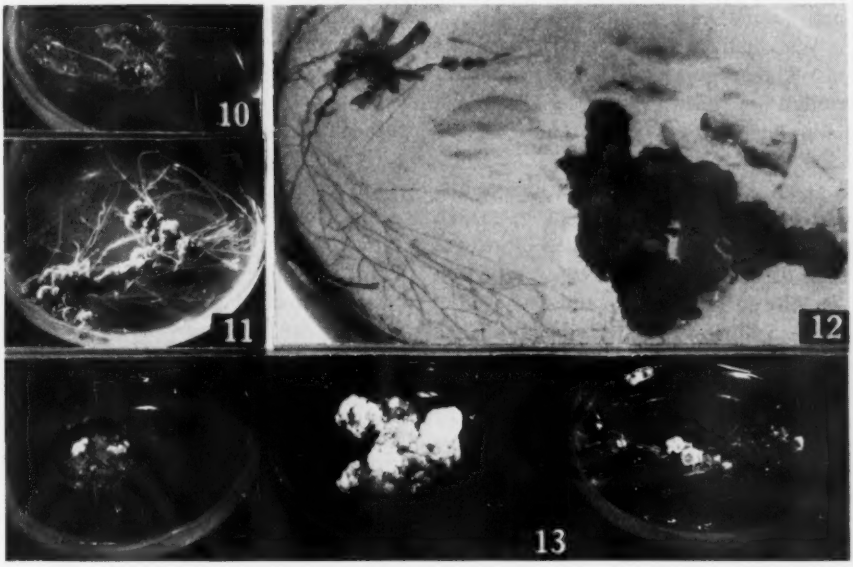
The evidence from these studies of the sunflower and carrot (7) indicates that IAA calluses differentiate when transferred to media deprived of this agent. Generally three to four passages of the IAA-induced calluses to similar IAA media and their subsequent removal to media free of IAA bring about complete differentiation in a large number of cultures, Prolonged growth of a tissue mass on IAA at the higher concentration level does not seem to facilitate development of shoots.

Cultures derived from fibrous roots and planted on Wt with IAA (0.1 mg/l) on April 14, 1949, were divided and transferred on June 21, 1949, to Wt CM. These cultures at first developed a few rosettes of leaves, and by August 17, 1949, a few fairly well developed plantlets were formed (fig. 8). In another series of cultures the calluses with their fragments of fibrous roots were subjected to several passages of IAA (5 mg/l) and then were planted on Wsf CM plus IAA (5 mg/l). Growth proceeded more abundantly under the influence of CM plus IAA, as shown for the carrot (1). The hyaline tissue masses formed large, opaque, nodular growths with several white roots which arose from the ventral surfaces of the tissue. On May 4, 1949, transfer was made to Wsf CM, and by August 17, 1949, each of a small number of cultures produced many plantlets (fig. 9).

Autoclaved coconut milk when added to basic media seems to stimulate growth of the tissue callus. Its effect seems to be greater, and growth becomes accelerated when it is combined with IAA. Coconut milk alone does not facilitate differentiation and more frequently seems to impede it. Plantlets formed on such a substrate, when compared with those formed on basic or carcinogenic media, appear weak and prostrate. VAN OVERBEEK (13) held that autoclaved coconut milk contains auxin apparently as distinct from an IAA-like substance. The present observation led to the belief that the IAA-like substance in a 10% concentration of coconut milk is so dilute that it fails to bring about typical IAA effects. Roots planted on CM have shown nodular epidermal proliferation. Constancy of the stimulating substances in coconut milk may vary with the source, age, size, and length of storage of the coconut.

N. GLAUCA × N. LANGSDORFFII.—Tissue cultures of this hybrid tobacco were started from segments of stems of non-"tumor"-bearing plants and from the spontaneous leafy overgrowths of the stems and peduncles. Frequently the

small "tumors" from the stem or floral parts of the plant, when seeded on standard medium, increased in size and formed green leafy shoots, with an abundance of fibrous roots. Stem segments, however, formed a callus with roots. In subsequent transplants both types of cultures assumed a uniform appearance, resembling a lateral stiped pileus. The upper surface of this structure, when actively growing, was frequently covered with a white tomentum; as growth became impaired, the tissue mass assumed a tawny color throughout. Fibrous roots from these cultures were excised and planted on various media.



FIGS. 10–13.—Cultures of tobacco hybrid *N. glauca* × *N. langsdorffii*. Figs. 10, 11, 13, ×4/9; Fig. 12, ×2. Fig. 10, fibrous root grown on Wt IAA (5 mg/l); started January 28, 1949, and photographed on March 16, 1949. Fig. 11, fibrous roots seeded on Wt CM, started as shown in figure 10. Fig. 12, callus development and differentiation of buds on roots from IAA culture grown on Wsf; transplanted April 25, 1949; photographed August 2, 1949. Fig. 13, growth of calluses on fibrous roots; *left to right*, Wsf, Wsf CM, and Wsf IAA (5 mg/l); seeded August 2, 1949, photographed September 22, 1949.

The fibrous-root cultures were initiated on January 28, 1949. The roots were derived from stem-segment cultures grown on Ws beginning August 18, 1948, and then passed to media with IAA (5 mg/l). The IAA-treated roots (fig. 10) produced small opaque, tawny-colored nodular masses covered with a thin white felt or tomentum. The roots made no linear increase. The roots planted on Wt with 10% CM (fig. 11), photographed March 16, 1949, produced a large, more active growth of nodules, with many new white roots. The larger nodules were tawny in color and heavily covered with a layer of white, feltlike tissue. Thus, for these roots coconut milk served as a better stimulus to callus formation than did IAA. In the sunflower, tobacco, and occasionally the carrot, fibrous roots produced small intumescences on CM, but none attained the proportions of

those developed on IAA. This variation in response between tobacco hybrid and the other species was at first considered due to the age (20 days old) of the IAA solution used in the preparation of the media. This supposition became untenable, since IAA solutions 60 days old were found to be capable of invoking IAA responses. When, on April 25, 1949, parts of the IAA calluses were planted on Wsf medium, however, the nodular masses grew in size. These were again divided and grown on Knop's and Wsf media with IAA and CM. Some of the calluses were retained on Wsf, and by August 2, 1949, had formed long roots, with small rosettes of leaves (fig. 12). In a small percentage of these cultures small green, budlike structures appeared from the surface of the calluses.

The calluses transferred to Knop's medium with IAA (5 mg/l) on June 6, 1949, were divided and transferred again on August 2, 1949, to Wsf, Wsf CM, or Wsf IAA. These consisted of callus bodies with few or no roots. Sample responses are shown (fig. 13) approximately 51 days after seeding. The central culture on CM shows an active tomentose-covered callus. Calluses on the standard medium Wsf (left) were small, composed of nodular masses with few patches of active tissue. In the culture on the right on Wsf IAA are seen two green buds on an old brown callus mass. Roots developed abundantly from this tissue. Small calluses on fibrous roots which developed on standard media with CM were studied. These were separated and planted on IAA and IAA CM. No differentiation of stem or leaves occurred, yet in a few cases the tissue masses produced a small number of roots.

The tissue mass of the tobacco hybrid is capable of forming roots and complete plantlets. The root under the influence of IAA is capable of producing tissue masses. Differentiation of the tissue mass to form plantlets is sporadic.

Summary and conclusions

1. Fibrous roots from tissue cultures of the sunflower (*Helianthus annuus* var. Russian) and tobacco (*Nicotiana affinis* and the hybrid *N. glauca* × *N. langsdorffii*) were grown *in vitro* in standard media to determine the effect of indoleacetic acid (IAA), chemical carcinogens (3,4-benzpyrene, 1,2,5,6-dibenzanthracene, and 20-methylcholanthrene), and coconut milk, all added singly or in combination, on these organs.

2. Growth of the fibrous roots was stimulated by the chemical carcinogens. There were linear increments with many branches formed in excess of those grown on basic media only.

3. Root cultures grown on basic media enriched with IAA failed to increase in length but formed hyaline, jelly-like calluses which turned dark brown in the sunflower and tobacco. The hybrid tobacco roots responded much more slowly and formed fewer calluses. Secondary roots appeared from these calluses.

4. Coconut milk induced no changes except in the hybrid tobacco roots where tawny-colored or tomentose-covered calluses were formed. Occasionally small, nodular calluses were observed on sunflower and *N. affinis* roots grown on media with coconut milk. Roots grown on media containing IAA combined with coconut milk produced dark-brown, compact nodular calluses in the sunflower and tobacco.

5. Calluses on these fibrous roots were excised and planted on standard media with the agents studied. The sunflower calluses consisted of large hyaline masses covered with roots. The *N. affinis* root

cultures and those of the tobacco hybrid formed tissue masses with roots. Subsequent transplants of these cultures to media deprived of IAA produced completely differentiated plantlets.

6. These observations lead to the conclusion that the tissue mass grown *in vitro* may produce organs (roots), and the organs may be induced to produce a tissue mass capable of differentiating complete plantlets.

Thanks are due Dr. E. J. BAUMANN, chemist, under whose direction the nutrient solutions used in these studies were prepared.

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EFFECT OF CERTAIN DYES ON THE GROWTH IN VITRO OF VIRUS TUMOR TISSUE FROM RUMEX ACETOSA[1]

LOUIS G. NICKELL

Introduction

This paper reports the effect of several dyes on growth of virus tumor tissue from *Rumex acetosa* L. grown in sterile culture. This series of investigations involves preliminary screening and selection of certain compounds for further, more exacting study on the basis of their effect on growth inhibition of the tumor tissue which is the virus-host complex.

TAKAHASHI (**22**) used the diaminotriphenylmethane dye, malachite green, in his study of the inhibition of virus increase. He postulated that enzyme systems indispensable for virus multiplication might be identified by the use of enzyme blocks because of the intimate association of virus reproduction with the metabolism of the host cell. Since such inhibition must take place *in vivo*, it would be necessary that the inhibitor not only be somewhat specific but also be active at a concentration nonlethal to the living host tissue. TAKAHASHI chose malachite green because it seemed to fulfil these conditions. It could penetrate living cells (**1**) and had no significant effect on the oxygen uptake of tobacco stem tissue at concentrations of 3.3 p.p.m. or lower (**5**).

By use of a detached-leaf-tissue technique (**21**), TAKAHASHI studied the effect of low concentrations of malachite green on the increase of tobacco mosaic virus in leaf tissue of *Nicotiana glutinosa* (**22**). Considerable inhibition of virus increase was shown at 2 p.p.m., and more at 4 p.p.m. At these concentrations there was little effect on the extracted virus *in vitro*. He concluded that "the inhibition of virus increase in living cells by low concentrations of malachite green appears to be due to its action as a block in enzyme reactions leading to virus formation."

In studying the physiology of the virus tumor tissue from *Rumex*, ability to inhibit increase of the virus would aid greatly in the study of virus-host relationships. Further, by destroying the virus completely, an excellent control tissue would be obtained.

Material and methods

The experimental tissue used was Black's original R_1 strain of wound-tumor tissue isolated from roots of *Rumex acetosa* L. (**2**). The basal medium was no. 24 of NICKELL and BURKHOLDER (**4, 14**) which is characterized by high levels of nitrate and phosphate. The dyes to be tested (table 1) were added to the basal medium in concentrations ranging from 0.000001 to 100 p.p.m. The pH values of all test media were adjusted to 4.5 before autoclaving. Other methods, as well as the cultural conditions, were essentially the same as those employed in previous studies with this material (**4, 12–16**). After 3 weeks, growth values were determined. Growth value as used here is a figure obtained

[1] This investigation was supported in part under a grant-in-aid by the American Cancer Society upon the recommendation of the Committee on Growth of the National Research Council.

by dividing the final weight by the original weight of inoculum. Each experiment was run in replicates of five, and an average was taken. Values expressed in this paper are the average of those taken from at least two experiments in close agreement. Results (figs. 1, 2) are expressed in percentage inhibition or stimulation based on the control (tissue grown on the basal medium only), computed by using the growth values.

Results

Eight dyes commonly used in histological and cytological work were included in this study. Under the experimental conditions employed no dye had any significant effect on the growth of the virus tumor tissue at concentrations of 0.001 p.p.m. and lower, with the exception of trypan blue (figs. 1, 2). Three dyes were found to be quite inhibitory to growth: methylene blue, crystal violet, and malachite green (fig. 1). The last two dyes caused complete inhibition of growth and death of the tissue at 10 p.p.m. and almost 60% inhibition of growth at 1 p.p.m. Neutral red caused a 20% inhibition of growth at 0.1 p.p.m., but concentrations of it

TABLE 1

Dye	Source	C.I. No.	% Dye	Certification no. or lot no.
Neutral Red	National Aniline and Chemical Co., Inc.	825	69	NX 4
Trypan Blue	Allied Chemical and Dye Corp.	477	.....	11003
Azure A	Allied Chemical and Dye Corp.	.....	92	NAz 14
Methyl Green	Allied Chemical and Dye Corp.	685	85	NG 26
Pyronin Y	Allied Chemical and Dye Corp.	741	32	NP 13
Malachite Green	Hartman-Leddon Co.	657	98	X-10-1
Methylene Blue	Allied Chemical and Dye Corp.	922	89	NA 28
Crystal Violet	National Aniline and Chemical Co., Inc.	681	95	NC 12

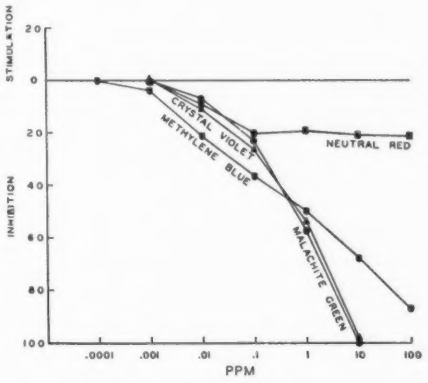

FIG. 1.—Inhibitory effect of certain dyes added to basal medium (no. 24) on growth of virus tumors of *Rumex*. Responses expressed as percentage inhibition or stimulation based on control tissue.

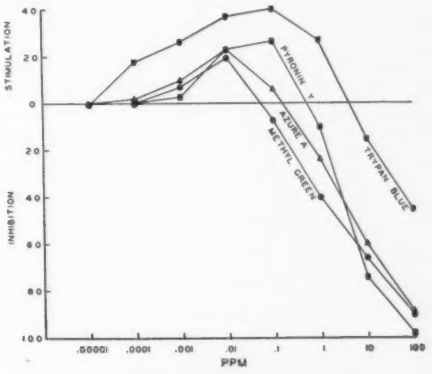

FIG. 2.—Growth responses of virus tumors to dyes added to basal medium (no. 24). Responses expressed as percentage inhibition or stimulation based on control tissue.

above this did not cause any change in the amount of inhibition (fig. 1). Four dyes—trypan blue, pyronin Y, azure A, and methyl green—were found to stimulate growth at concentrations of 0.01 and 0.1 p.p.m. (fig. 2). Trypan blue caused stimulation over a greater range—from 0.0001 to 1 p.p.m.—than did the other three dyes, with maximum stimulation of about 40% at 0.01–0.1 p.p.m. Methyl green and azure A caused stimulation of growth at 0.01 p.p.m. of about 20% over the control. Pyronin Y caused stimulation of about 25% at 0.01–0.1 p.p.m.

Discussion

Of the dyes tested, trypan blue most effectively stimulated growth of the virus tumor tissue, while malachite green and crystal violet most effectively inhibited it. By interpolating at a value of 2 p.p.m. on the malachite green curve in figure 1, it seems probable that this concentration would cause about an 80% inhibition of growth.

These dyes did not permanently affect growth of the virus tumor tissue. When viable tumor material grown on medium containing dye was placed on the basal medium alone, its growth rate returned to normal. This has been demonstrated with a dye representative of each type of effect: malachite green, neutral red, and trypan blue.

The effect of these dyes on the color of the tumor tissue is another interesting observation. An early article (**4**) on *in vitro* culture of this material reported that the green-brown ratio of color was proportional to the rate of tissue growth—the better the growth, the higher the ratio, and vice versa. This has proved true, with two exceptions, in all subsequent experiments using this tissue. When starch was used as a carbon source or when aspartic acid was present in the medium, the tissue was excessively green according to the standard used throughout this work (**12, 15**). The effect of dyes on tissue color is also an exception. The presence of a dye changed the color of the virus tumor tissue—from that of tissue grown on the basal medium—*only* at those concentrations causing death of the tissues. This includes both inhibitory and stimulatory concentrations. This lack of color effect might be interpreted as a result of the selective action of the dyes against the virus and not against the host tissue which allowed the host tissue to remain relatively undisturbed. To check this hypothesis, experiments are in progress to test the dyes against normal *Rumex* tissue.

It is of particular interest that the four dyes found to stimulate growth of the virus tumor tissue are all used as specific stains for nucleic acids or nucleoproteins: trypan blue (**9–11, 18–20**), pyronin Y (**3, 7, 8**), azure A (**6**), and methyl green (**3, 7, 8, 17**).

Summary

1. The effect of various dyes on the growth of virus tumor tissue from the roots of *Rumex acetosa* L. was tested by adding the dyes to a basal medium and culturing the tissue on these substrates for 3 weeks.

2. Methylene blue, crystal violet, and malachite green inhibited growth at concentrations above 0.01 p.p.m. and had no effect at lower levels. Neutral red was slightly inhibitory (about 20%) at 0.1 p.p.m. and higher, but higher concentrations did not result in greater inhibition. Trypan blue, pyronin Y, azure A, and methyl green caused a stimulation of growth at concentrations of 0.01 and/or 0.1 p.p.m.

3. The effect of these dyes on the growth of the tumor tissue was not

permanent. When the tissue was placed on the basal medium, after having been grown on a medium containing a dye, the growth rate returned to normal.

4. Unlike most other compounds, the dyes had no effect on the color of the tumor tissue, even when used at inhibitory concentrations.

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HYPERTROPHY FROM THE UREDIAL STAGE OF BEAN RUST

C. E. YARWOOD AND MORRIS COHEN

Introduction

Although hypertrophy from the pycnial, aecial, and telial stages of many rusts is common, it has rarely been recorded for the uredial stage. In 1929 ARTHUR (1) indicated that hypertrophy from uredial stages was unknown, but in 1934 (2) he noted the association of the uredial stage of certain species of *Ravenelia* with woody galls and witches'-brooms. LUDWIG (4) illustrated the uredial stage of *Uredo notabilis* Ludw. on galls on *Acacia notabilis* F. v. M. RICE (6) reported hypertrophy of individual cells and nuclei of corn infected by the uredial stage of *Puccinia sorghi* Schw. but indicated no general overgrowth. The uredial stage of bean rust, *Uromyces phaseoli typica* Arthur, has been maintained in Berkeley in greenhouse cultures and on detached leaves for 12 years without host hypertrophy having been observed heretofore. The conditions for its occurrence may therefore be of interest, especially since it seems probable that a similar situation may exist with other rusts.

Observations

LOCALIZED HYPERTROPHY.—Hypertrophy associated with bean-rust infections has been most conspicuous when vegetatively immature primary leaves of bean, *Phaseolus vulgaris* L. var. Pinto, inoculated to give single or multiple infections in localized areas, were detached and incubated on sucrose solution. In the procedure a finely pointed wet brush was touched first to a mass of urediospores on an infected leaf and then to the lower surface of a healthy leaf on a potted greenhouse plant. The plant bearing the inoculated leaf was incubated overnight in a dark moist chamber. It was then removed from the chamber and left in the greenhouse until any free water on the leaves had evaporated. The inoculated leaf was then detached at the pulvinus and placed with its lower surface in contact with 7 cc. of 8% sucrose solution in a closed Petri dish. The cultures were kept at about 22° C. in a laboratory receiving light through a northwest-facing window. The infected areas appeared as concave or convex bulges in about 6 days (fig. 1). Hypertrophy from localized infections has occurred on leaves on entire plants but was not so striking as on detached leaves. Little or no local hypertrophy was seen when numerous infections (five or more per square centimeter) were distributed evenly over the entire leaf area, or with infections on almost full-grown or mature leaves, or with detached leaves on water instead of sucrose solution.

In one trial not yet repeated, leaves of the bean variety Bountiful were detached and placed on water 1 day after inoculation. At 8 days after inoculation infection ranged from five to thirty lesions per square centimeter. All lesions showed conspicuous convex or concave hypertrophy, but most pustules showed no sporulation. Similar leaves on sucrose solution showed sporulation of all pustules but showed hypertrophy only on portions of the leaf not in contact with the sucrose solution. It therefore appears that marked differences exist be-

tween bean varieties with respect to hypertrophy from bean rust.

To determine the nature of the hypertrophy on Pinto bean, comparable areas from opposite halves of the same leaf, one area normal, the other convexly hypertrophied 8 days after a local inoculation, were examined microscopically after clearing in a saturated chloral hydrate solution. Twenty random palisade areas, even in the absence of rust mycelium.

By means of sections cut 7 days after inoculation and cleared in chloral hydrate, leaf thickness and cell dimensions were measured in the nonsporulating, mycelium-invaded, and adjacent noninvaded, hypertrophied region and in the tissues 1 mm. beyond the mycelium (table 1). Hypertrophy, as revealed by

FIG. 1.—Hypertrophy resulting from bean rust as seen from upper surface. Plants seeded on November 6, 1949, inoculated on November 13, leaves placed on 8% sucrose on November 14, and photographed on November 23. On right leaf are two single-pustule infections, one double-pustule infection, and two multiple-pustule infections in convex hypertrophied areas, and one multiple infection with combined concave and convex hypertrophy. On left leaf are two multiple-pustule infected areas with concave hypertrophy.

cells averaged 15.8 μ in their cross-sectional diameter for the healthy portion and 20.0 μ for the hypertrophied area. The smallest and the largest palisade cell in each of ten microscopic fields averaged 9.7 and 21.3 μ, respectively, for the healthy portion and 12.5 and 28.6 μ for the rusted portion. The greatest diameter of the largest upper epidermal cell in each of five microscopic fields averaged 63 μ for the normal and 76 μ for the rusted portion. The palisade tissue of the hypertrophied areas had smaller intercellular spaces than that of the normal leaf thickness and either cell dimensions or cell volume, was less the greater the distance from the center of infection. No evidence of recent cell division was observed. It is concluded that the macroscopically apparent hypertrophy is primarily the result of microscopic hypertrophy of the leaf cells. The forcing-apart of cells by the intercellular mycelium may be an additional factor in the macroscopic hypertrophy of the invaded zone.

INCREASE IN LEAF SIZE OF TWIN LEAVES RESULTING FROM RUST INFECTION.—When one of a pair of opposite

primary leaves—usually closely comparable in size and growth rate—was evenly inoculated over its entire surface, hypertrophy of the infected leaf was observed. The dimensions of the leaves were measured before inoculation, at various times after inoculation, and usually until growth ceased. Area was calculated as length×width/1.35; 1.35 is the average quotient when the planimetered area of primary leaves of Pinto beans is divided into the product of length and width. some cases, where an infection dosage series was studied, satisfactory pustule counts were unobtainable after heavy inoculations and were consequently estimated by extrapolation of the straight line relating the logarithm of the number of original infections per square centimeter (based on the lower values which could be counted) to the logarithm of relative inoculum concentration.

After growth of the uninoculated leaves had ceased, that of the inoculated leaves

TABLE 1

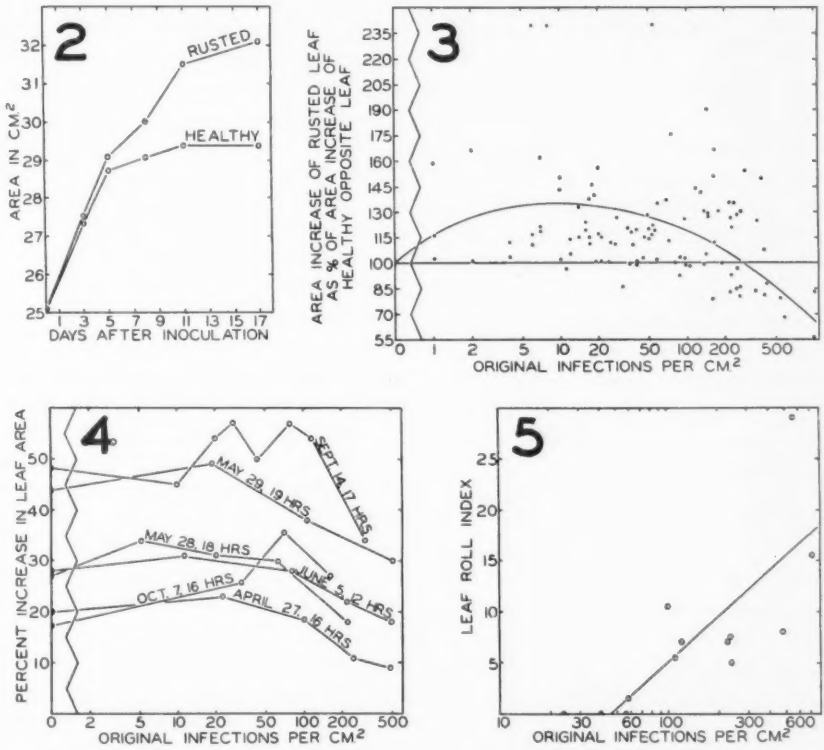
LEAF THICKNESS AND CELL SIZE IN MACROSCOPICALLY HYPERTROPHIED TISSUE INFECTED WITH BEAN RUST

Zone of measurement	Average leaf thickness in microns	Average cell dimensions in microns (10 cells measured)				
		Thickness of upper epidermis	First palisade layer	Second palisade layer	Spongy mesophyll (max. diam.)	Thickness of lower epidermis
Mycelium-invaded, nonsporulating	227*	21	55×16	37×19	31	20
Adjacent, uninvaded	210†	18	46×14	26×16	29	17
Uninvaded, 1 mm. beyond	183†	15	48×11	25×12	21	14

* Average of 49 measurements.
† Average of 13 measurements.

The number of pustules per square centimeter was counted when they became clearly apparent but is expressed as the number of infections per unit area of leaf at the time of inoculation—a figure calculated by multiplying the observed number of pustules per square centimeter by the ratio of the area of the leaf at the time of counting to the area of the leaf at the time of inoculation. This conversion was made because, as will be shown, some effects of rust infection are expressed in the first few hours after inoculation, while by the time the infection is counted the leaf may have increased in area by several hundred per cent. In continued, presumably as a result of infection (fig. 2). No distortion of the leaves resulted from this type of hypertrophy, except that in some cases heavily infected leaves, inoculated when quite young, rolled down parallel to the main leaf axis 5–10 days after inoculation, an effect somewhat the opposite to that induced by rust infection in the first 24 hours after inoculation. The original differences among plants in size of their primary leaves when inoculated could not be related to clear-cut differences in hypertrophy from rust, except that hypertrophy resulted only on an expanding leaf.

An attempt was made to determine whether there was a relation between infection dosage and the amount of hypertrophy. Data of all tests (106 paired comparisons, fig. 3) show that there was little difference in the effects produced by rust dosages ranging from 1 to 200 original infections per square centimeter and that in this range the increase in leaf size attributable to rust ranged up to



FIGS. 2–5.—Fig. 2, rate of growth of twin bean leaves, one inoculated with rust, the other not. Each value is average of eight leaves. Degree of infection for rusted leaves averaged forty-six pustules per square centimeter of original leaf area. Plants seeded on December 8, inoculated on December 23, 1948. Average increase in growth of rusted leaves illustrated here is about 9%—considerably less than average of all trials plotted in figure 3. Fig. 3, relation between density of rust infection and area of infected bean leaves relative to healthy twin leaves after average infection period of 10 days. Each value is from one pair of leaves. Fig. 4, relation of density of rust infection to growth in area of bean leaves when both primary leaves were similarly inoculated on dates shown and measured after indicated incubation periods. Each value is average of four plants. In every case, at some level of infection below 200, and usually below 100, original infections per square centimeter, there is an increase in growth rate while a decrease occurs at higher infection levels. Fig. 5, relation of density of rust infection to amount of leaf-roll. Leaf-roll index was calculated as:

$$\frac{\text{Flattened width of leaf minus rolled width of leaf}}{\text{Flattened width of leaf}} \times 100.$$

Rolled width of leaf was measured in rolled condition as distance between leaf edges at widest part of leaf. For three trials represented here, measurements were made 16 hours after inoculation in two trials and after 23 hours in another. Index of leaf-roll increases with density of original infections.

140% and averaged about 25%. At disease levels represented by more than 500 original infections per square centimeter, leaf growth was reduced by the rust infection.

INCREASE IN LEAF SIZE RESULTING FROM INOCULATIONS OF ENTIRE PLANTS.—When entire bean plants, whose primary leaves had not ceased growth, were inoculated with rust, infection appeared almost exclusively on the laminae of the leaves. Reduced growth following heavy inoculations was apparent in about 8 days, and increased growth resulting from any level of infection at any time was not clearly apparent in the absence of measurements. In comparing this situation with that of experiments with opposite leaves, it must be remembered that random or even paired entire plants are much more variable than twin leaves. The use of paired plants, with one inoculated and one not, might seem the most delicate type of comparison which can be made to study the effect of rust infection on entire plants. It soon became apparent, however, that infection level (original infections per unit area of leaves) determined whether there was an increase or decrease in leaf growth as a result of the rust infection; and, as we wished to study a wide range of infection levels, it would have been necessary, if paired plants were to be used, to maintain the same number of controls as treated plants. The compromise adopted was to maintain as many controls—four—as there were replications of each treatment. Measurements of leaf size were made just before and at various intervals following inoculation. The first measurements after inoculation (fig. 4), made at the time of removing the plants from the dark, moist, incubation chambers, show that for infection levels ranging from 0 to 100 original infections per square centimeter, area increases resulting from rust infection averaged about 10%. With 200–800 original infections per square centimeter there was an average decline of 30% in leaf growth. In all six trials there was an increase in leaf growth at low infection levels and a decrease at high infection levels, though there were some discrepancies for certain groups of values.

LEAF-ROLL RESULTING FROM BEAN RUST.—A transitory upcurl of primary bean leaf blades during the early stages of infection with rust may be a type of hypertrophy. When young leaves heavily inoculated on the lower surface were incubated in a dark moist chamber, the blades usually curled up, somewhat as in virus leaf-roll of potato, while uninoculated leaves on the same or different plants in the same environment, but sprayed with water, remained approximately flat. In some cases the upward roll was primarily at right angles to the midvein of the leaf, but in most cases the most conspicuous roll was parallel to the main vein. On several occasions this leaf-roll was not apparent 10–20 hours after inoculation but did appear when the same plants were left in the moist chamber until 15–24 hours after inoculation. When inoculated plants were transferred from the dark moist chamber to the greenhouse bench, the curled leaves gradually returned to their normal flat condition in 12–48 hours but, if left in the dark moist chamber, did not recover in 84 hours. Leaf-roll was clearly associated with high levels of rust infection in several tests (fig. 5), but the data in figure 5 include only tests in which a series of infection dosages was under study and do not include the most dramatic cases observed where only one level of infection was maintained. In one case the leaf edges rolled up so that they overlapped

by several millimeters. In one test primary leaves on plants 8 days from planting showed more pronounced rolling than did leaves of 13-day-old plants. In another trial inoculations on the upper leaf surface resulted in no leaf-rolling, either up or down, while inoculations on the lower surfaces of the opposite leaves on the same plants resulted in pronounced upward rolling, a phenomenon which, because host penetration occurs through the stomata, may be related to the greater number of stomata on the lower than on the upper surface.

Although the phenomenon of leaf-roll resulting from rust infection was most pronounced on leaves whose area increase was reduced by high levels of infection (cf. figs. 3 and 5), it still seems that it may be explained by the temporarily greater growth of the lower (inoculated) than the upper (uninoculated) side of the leaf. A possible explanation is that infection with bean-rust injures, or at least has a physiological effect on, the guard and epidermal cells, as has been observed with *Puccinia* on wheat (3), *Gibberella* on corn (5), and *Peronospora* on onion (7). This injury might increase the permeability and water uptake of these cells with resultant expansion of cell size while in the moist chamber, and this in turn might cause the leaf to curl up. Evidence in support of this view is that the leaves returned to normal shape when placed in the greenhouse environment. The fact that leaves inoculated on the upper surface did not curl down and that leaf-roll has resulted from infiltrating detached leaves with water in the absence of rust inoculation are evidence against the hypothesis. In several trials designed to study leaf-roll, no rolling resulted from any treatment; thus the conditions for its occurrence are imperfectly understood.

At 8–25 days after inoculation or 7–24 days after they had recovered from the initial leaf-roll associated with the early stages of infection, rusted leaves sometimes curled up and sometimes down. It seemed that for this late manifestation of leaf-roll, a rather light infection (around 2–20 pustules per square centimeter) caused the leaves to roll down; but the leaf-roll associated with late stages of infection was not quantitatively studied.

Discussion

If our interpretations are correct, the important question which remains unanswered is: Whence comes the energy for the increased growth which results from rust infection? In addition to the increased growth in area and volume described here, considerable localized increase in dry weight also results from rust infections (8). A previous explanation (8) has been that the local increase in dry weight results from decreased translocation; this could explain all the results of the present study. Recent unpublished results indicate, however, that there may be an actual increase in the total dry weight of entire plants resulting from rust infection and that this increase, which is temporary, results from a temporary increase in the rate of photosynthesis. It is hoped to present these data in a future publication.

Summary

1. Hypertrophy during the uredial stage of bean rust (*Uromyces phaseoli typica* Arthur) on bean (*Phaseolus vulgaris* L. var. Pinto) was manifested (*a*) by concavities or convexities in the areas of localized light or heavy inoculations of primary leaves, (*b*) by visible increases in the total growth of infected primary leaves when compared with that of their respective opposite healthy leaves, and (*c*) as measurable increases in total

growth of leaves when entire plants were inoculated.

2. In the second type of comparison (*b*) the increase in growth resulting from rust infection was most pronounced in the later stages of leaf growth, but in the third type (*c*) it was clearly measurable in as little as 12 hours after inoculation.

3. In the last two types of comparison (*b*, *c*) hypertrophy occurred only where there were about 200 or less original infections per square centimeter of leaf area; more infections than this usually reduced the rate of leaf growth.

4. The transient upcurling of leaves following heavy inoculation of the lower epidermis may also be a type of hypertrophy occurring under conditions of high humidity.

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EFFECTS OF SULFUR DEFICIENCY ON GROWTH AND METABOLISM OF TOMATO

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 621

SCOTT V. EATON

Introduction

In this series of investigations the writer has studied the effects of sulfur deficiency on three plants: soybean (**2**), sunflower (**3**), and black mustard (**4**). Results were qualitatively similar for all the plants. NIGHTINGALE *et al.* in an excellent paper (**6**) reported the effects of deficient sulfur on the metabolism of tomato plants grown at New Brunswick, New Jersey. One important difference was that reducing sugars were high in minus-sulfur tomato stems as compared with plus-sulfur ones, while in sulfur-deficient soybean, sunflower, and black mustard stems reducing sugars were low.

The present work was undertaken to compare the effects of sulfur deficiency on tomato plants grown in Chicago with those grown in New Jersey (**6**) and with the other plants of this series of studies.

Material and methods

Tomato plants (*Lycopersicon esculentum* Mill.) var. Marglobe were grown in pure quartz sand by essentially the same methods as previously described (**5**) except that the concentration of major elements of the nutrient solution was that used in sulfur-deficiency work on the sunflower (**3**). Seed was planted in the sand in a flat on April 26, 1950. In order

to secure enough tissue for analysis, the plants were supplied with a complete nutrient solution diluted one-half for one week, then given full strength until May 12. They were then transplanted to quartz sand in 2-gallon glazed crocks, and two-thirds of the plants were given a minus-sulfur nutrient solution, the others being kept on complete nutrient. The effects of sulfur deficiency were so drastic, however, that it was necessary on June 2 to supply sulfur to the sulfur-deficient plants. They were given complete nutrient solution until June 8, when minus-sulfur treatment was resumed.

Methods of sampling, extraction, and chemical analysis were essentially the same as previously described (**5**). Sulfur was determined by the Official Methods (1). There were two harvests, June 15 and 30. The number of minus-sulfur and plus-sulfur plants were, for the first harvest, 148 and 71, respectively; for the second, 94 and 48. Only stems were analyzed.

Results

The tomato plant seems very sensitive to sulfur deficiency, and symptoms develop rapidly. Nine days after beginning the no-sulfur treatment, leaves of minus-sulfur plants were lighter green than those of plus-sulfur plants; there was much more anthocyanin in stems and leaves of the former than of the latter. The youngest leaves developed anthocyanin first, and appearance of the pigment in them was a good index of the onset of sulfur deficiency. At this time plants of both series were small with only one or two well-formed leaves, and it was impossible to determine the gradation of chlorosis of minus-sulfur plants. It then became necessary to allow additional leaves to develop during a 6-day period on complete nutrient, during which interval sulfur-deficient leaves became almost, if not quite, as green as plus-sulfur ones. Then on resumption of no-sulfur treatment chlorosis became evident in most plants in 4 days. Upper leaves became chlorotic first, and there was a regular increase in greenness toward the base of the plant. There was also somewhat more anthocyanin in minus-sulfur plants than in those amply supplied. In preliminary work much older plants about 10 inches tall with seven or eight rather large leaves became chlorotic in less than 2 weeks and showed the same gradation of chlorosis.

Other symptoms of a deficient supply of sulfur were small leaves, their stiff texture, and thin stems. Small size of leaves in comparison with those of complete-nutrient plants was evident about 9 days after first application of minus-sulfur solution, but the stem difference did not appear until about 19 days after treatment began. Sulfur-deficient plants were on the average considerably shorter than plus-sulfur ones, yet they elongated remarkably and in many cases were eventually almost as tall as the latter plants, although they weighed much less. Figures 1 and 2 show the relative size and appearance of plants of the two series.

Table 1 gives data for fresh weight, percentage water, and percentage dry weight of the stems. Minus-sulfur stems weighed much less than plus-sulfur ones. Weights of leaves and roots were also taken but, since these data varied in a manner similar to those for the stems, are not given. Water and dry-weight percentages of stems of the two groups did not vary greatly.

It should be emphasized that, since the minus-sulfur plants were put back on complete nutrient for 6 days beginning on June 2, the weight data do not indicate the true effects of sulfur deficiency

on growth of plants. Size differences would have been even greater if minus-sulfur plants had not been given this additional sulfur. On the other hand, data of the later tables indicate that sulfur deficiency was just beginning to affect the chemical constitution of the plants at the first harvest, although chlorosis of

FIGS. 1, 2.—Effect of sulfur deficiency on tomato; minus-sulfur plants on *left*, plus-sulfur on *right*. Fig. 1 (*above*), at first harvest, 50 days after planting of seed. Fig. 2 (*below*), at second harvest, 65 days after planting of seed.

leaves of most plants was evident. As stated above, during the 6-day period on complete nutrient, minus-sulfur leaves became almost as green as plus-sulfur leaves.

TABLE 1

DATA ON WHOLE STEMS

Solution	Fresh weight (gm/100 plants)	Percentage water	Percentage dry weight
	First harvest		
Minus S.	198	94.7	5.3
Plus S.	631	93.9	6.1
	Second harvest		
Minus S.	958	91.2	8.8
Plus S.	2432	91.4	8.6

fur-deficient plants were lower than those of plants grown with complete nutrient. These ratios were, for the first and second harvests, 5.679 and 5.529, respectively, for minus-sulfur plants; and 56.998 and 79.695, respectively, for the plus-sulfur plants.

Chemical constituents of the stems were figured on both dry-weight and wet-weight bases, but only the former data are given. Since stems of the two series differed little in percentage dry weight, comparisons of chemical data for minus-sulfur and plus-sulfur stems are similar for the two bases.

There was a higher percentage of soluble solids in minus-sulfur than in plus-sulfur stems at the first harvest, but the difference was reversed at the second harvest (table 2). Differences in stems of the two series in total nitrogen, soluble nitrogen, and insoluble nitrogen were small at the first harvest. At the second harvest there was almost twice as much soluble nitrogen and considerably more total nitrogen in the minus-sulfur stems as in the plus-sulfur ones. On the other

TABLE 2

SOLUBLE SOLIDS AND NITROGEN FRACTIONS
(PERCENTAGES ON DRY-WEIGHT BASIS)

Solution	70% alcohol-soluble solids	Total N	70% alcohol-soluble N	70% alcohol-insoluble N	Ammonia	Amides	Nitrates
	First harvest						
Minus S.	35.2	3.72	2.44	1.28	0.056	0.099	1.904
Plus S.	33.0	3.70	2.49	1.21	0.028	0.081	1.874
	Second harvest						
Minus S.	34.0	3.17	2.44	0.73	0.361	0.180	0.945
Plus S.	35.7	2.22	1.29	0.93	0.049	0.066	0.716

ones. Weight differences of stems of the two groups at first harvest give little indication of differences in chemical composition.

Sulfur deficiency affected tops more than roots, so that top-root ratios of sul-

hand, insoluble nitrogen was higher in the latter stems.

Table 2 also compares percentages of ammonia, amides, and nitrates in stems of the two sets of plants. Even at first harvest minus-sulfur stems were higher in all these fractions. Differences were much greater at second harvest. Minus-sulfur stems contained more than seven times as much ammonia and almost three times as much amides as the plus-sulfur ones. There was twice as much ammonia as amides in the former stems. This is an unusual situation even in plants suffering from a mineral deficiency and should be investigated further.

TABLE 3

CARBOHYDRATE FRACTIONS
(PERCENTAGES ON DRY-WEIGHT BASIS)

Solution	Total sugars	Reducing sugars	Sucrose	Starch
	First harvest			
Minus S....	1.74	1.12	0.62	0.79
Plus S.....	2.03	1.44	0.59	0.60
	Second harvest			
Minus S....	6.17	3.67	2.50	2.56
Plus S.....	13.27	11.33	1.94	0.99

At the first harvest sulfur-deficient stems were somewhat higher in starch, contained about the same amount of sucrose, but were lower in total and reducing sugars than the complete-nutrient stems (table 3). Differences were accentuated at the second harvest. The former stems contained almost three times as much starch, less than one-third as much of reducing sugars, and less than one-half as much of total sugars as the latter ones. The more complex carbohydrates—starch and sucrose—accumulated in the minus-sulfur stems, but the simpler reducing sugars were lower in them.

Effects of sulfur deficiency seemed to be just beginning at the first harvest. Low sulfur supply caused chlorosis of leaves, development of anthocyanin, and accumulation of starch, sucrose, and the soluble nitrogen fractions—ammonia, amides, and nitrates—but a smaller amount of reducing sugars in comparison with plants grown with complete nutrient solution. At the first harvest leaves of most but not all minus-sulfur plants were chlorotic, but the amount of anthocyanin then differed little in plants of the two series. The chemical differences mentioned above were present but were small except in the case of ammonia (tables 2, 3).

Sulfur determinations were made on stems from the second harvest only. Percentages of total sulfur, insoluble sulfur, and soluble sulfur were 0.358, 0.140, and 0.218, respectively, for the minus-sulfur stems and 0.522, 0.141, and 0.381 for the plus-sulfur stems.

Discussion

SUMMARY OF EFFECTS OF SULFUR DEFICIENCY

In previous work of this series studies have been made of the effects of sulfur deficiency on three plants: soybeans (2), sunflower (3), and black mustard (4). The paper on mustard contains a discussion of the effects of sulfur deficiency on plants in general.

The above plants were uniform in the major symptoms of a deficient supply of sulfur. These were chlorosis of leaves, smaller leaves, and thinner stems as compared with plus-sulfur plants. There was increasing greenness of leaves from top to bottom of the soybean and mustard plants. This was not noticed in the sunflower, probably because the onset of de-

ficiency symptoms was quick and drastic. All leaves were yellow. Closer observation would probably have revealed the same gradation of chlorosis as in the other plants. Stem elongation was prominent, especially in the soybean and sunflower.

Starch and the soluble nitrogen fractions—ammonia, amides, amino acids, and nitrates—accumulated in minus-sulfur stems, but such stems were lower in reducing sugars than the complete-nutrient stems. Sucrose was variable, being high in sulfur-deficient mustard stems but lower in minus-sulfur soybean and sunflower stems than in plus-sulfur ones.

The chemical data were interpreted as indicating that two opposing factors affected chemical constitution of the sulfur-deficient stems—proteolysis and lessened reducase activity. Proteolysis accounted for the accumulation of ammonia, amino acids, and amides, although part of the large amount of amino acids and amides was probably due to a deficiency in the amount of sulfur needed for formation of proteins from amino acids. Proteolysis also caused a decrease in reducing sugars in all the above plants and of sucrose in soybean and sunflower. Lessened reducase activity favored accumulation of starch in all plants and of sucrose in mustard. Usually all carbohydrates decrease in proteolysis and accumulate when there is interference with the reduction of nitrates. With both factors affecting the plants at the same time, proteolysis caused a decrease in the stems of all plants of reducing sugar, and of sucrose in soybean and sunflower; poor reducase activity, an increase of starch in stems of all plants, and of sucrose in mustard.

Data of the present study showed that the effects of sulfur deficiency on tomato plants grown in Chicago were similar to those previously found for soybean, sunflower, and mustard. The deficiency symptoms were similar. Chlorosis of leaves was prominent, upper leaves becoming chlorotic first. Starch, sucrose, total soluble nitrogen, and the soluble nitrogen fractions—ammonia, amides, and nitrates—were high, and reducing sugars were low in the sulfur-deficient stems as compared with the complete-nutrient ones. Amino acids were not determined, but they probably also accumulated, for concentrations of amino acids and amides in plant tissues usually run more or less parallel.

The results of NIGHTINGALE *et al.* (6) for tomato plants grown at New Brunswick, New Jersey, were similar to those of the present work in that starch, sucrose, and soluble nitrogen fractions accumulated in minus-sulfur stems as compared with complete-nutrient stems. There were two main differences. They found that the lower leaves became chlorotic first and that reducing sugars accumulated in sulfur-deficient stems along with starch and sucrose, while, as mentioned above, in tomato plants grown at Chicago, chlorosis developed first in the upper leaves, and reducing sugars were low in the minus-sulfur stems. NIGHTINGALE *et al.* recognized that proteolysis usually causes a decrease in all forms of carbohydrates and stated that an explanation of the discrepancy was not apparent.

POSSIBLE EXPLANATIONS OF DIFFERENCES IN EFFECTS OF SULFUR DEFICIENCY

NUTRIENT SOLUTION.—The minus-sulfur solution used by NIGHTINGALE *et al.* was higher in phosphorus and their plus-sulfur one higher in both potassium and phosphorus than those used in the present study. However, since all the nutrient solutions probably contained an

abundance of all essential elements except sulfur, variations in their composition probably did not account for the differences in results in the two investigations. In the series of studies conducted by the writer the nutrient solutions varied considerably in composition, yet sulfur deficiency affected all the plants similarly.

KIND AND AGE OF PLANTS.—The same variety of tomato plant was used in both studies, but pure-line seed was not used in the present experiment and apparently was not in the work of NIGHTINGALE *et al.*, so that the actual nature of the variety may have differed. However, since in the writer's work sulfur deficiency affected similarly plants as widely separated taxonomically as the soybean, sunflower, and black mustard, varietal variations in tomato probably did not account for the differences in the results of the two studies.

Age of plants at the beginning of no-sulfur treatment and at the harvests may have differed. No comparison can be made in this respect, for NIGHTINGALE *et al.* did not give the age of their plants at the start of nutrient treatment. However, age differences probably did not affect the results obtained. In preliminary work plants 12 days old at the beginning of no-sulfur treatment and analyzed 43 days later gave the same qualitative results as to total sugars and reducing sugars as plants 47 days old at the start of minus-sulfur treatment and harvested 41 days later.

ATMOSPHERIC CONDITIONS.—It is well known that variations in these conditions, especially temperature and light intensity, affect the chemical composition of plants. The above-mentioned differences in the effect of sulfur deficiency on tomato plants grown at New Brunswick, New Jersey, and Chicago were probably due to such variations.

Summary

1. Symptoms of sulfur deficiency developed quickly in tomato plants. They were: chlorosis of leaves, the upper leaves becoming yellow first, anthocyanin development in leaves and stems, small leaves with stiff texture, and thin stems.

2. Minus-sulfur stems were high in starch, sucrose, total soluble nitrogen, and the soluble nitrogen fractions—ammonia, amides, and nitrates—but low in reducing sugars as compared with plus-sulfur stems.

3. The above results agreed well with those of previous studies by the writer on the effects of sulfur deficiency on soybean, sunflower, and black mustard and in most respects with data of NIGHTINGALE *et al.* for tomato plants grown at New Brunswick, New Jersey. Their results differed in two main respects from those obtained by the writer. They found that the lower leaves became chlorotic first and that reducing sugars accumulated in minus-sulfur stems along with other carbohydrates.

4. It is concluded that variations in atmospheric conditions probably accounted for differences in effects of sulfur deficiency on tomato plants grown at Chicago and at New Brunswick.

5. In the discussion sections of the present paper and of other papers by the writer (**2**, **3**, **4**), especially the one on black mustard (**4**), are found summaries with interpretations of the effects of sulfur deficiency on plants in general.

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EFFECT OF PRE-ILLUMINATION ON THE RESPONSE OF SPLIT PEA STEMS TO GROWTH SUBSTANCES[1]

MARTHA KENT AND WILLIS A. GORTNER

Introduction

WENT (9) developed a unique laboratory method for detecting plant growth-regulator activity in chemicals, using the appearance of inward curvatures in split, etiolated pea stems as a criterion for activity. Many workers, however, including WENT, have noted a serious drawback to the use of this technique in that there is frequently a considerable variation in response to a given chemical from day to day.

JOST and REISS (2) reported that indoleacetic acid in a concentration of 10 p.p.m. failed to induce curvature on one day but on the next day brought about a very pronounced curvature. WENT (10) stated that "in about half the cases no curvatures, in the other cases weak to good pea tests were obtained after infiltration. . . . Other discrepancies are the apparent activity of phenyl butyric acid on some days, and its complete inactivity on others."

THIMANN and SCHNEIDER (5) noted that on peas grown "in the dark room with occasional red light" the absolute curvatures varied considerably from experiment to experiment. In discussing the differences in relative activities of given auxins on different days, they (6) stated: "Such variations are fairly large, and, as may be seen, introduce both quantitative and qualitative differences. The causes are obscure but must comprise some superficially minor differences in the conditions or the treatment of the plants."

VAN OVERBEEK and WENT (7) considered an advantage of the pea test to be that there is "no lack of sensitivity, if occasionally exposed to small amounts of white light." Although they did not mention the type or amount of illumination during growth of the seedlings in their routine work, they stated that "there is considerable variation in the sensitivity of peas on different days. . . . These variations are not due to a lack of uniformity of the material nor to changes of temperature, humidity, or dark room illumination; they depend on as yet uncontrollable external factors. . . . The same peas, or the same internodes even, may give different reactions on successive days."

In attempting to find an explanation

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for frequent poor responses in the pea test, we have found that the curvature response of the split pea stems to a growth substance is directly related to previous exposure to red or white light. WENT (11) and PARKER *et al.* (3) have recorded the effects of various wave lengths and intensities of light on the growth of pea seedlings, but not in relation to their sensitivity in the pea test.

Material and methods

Seeds of *Pisum sativum*, variety Alaska, were soaked in water for 4–6 hours and then germinated between wet paper towels for 3 days. They were then transferred to a perforated porcelain or lucite plate supported above a shallow dish of water. In this way the seed was supported on the plate and the root allowed to reach the water through the hole. The water was changed daily to avoid a root rot to which the plants were especially susceptible on the fourth and fifth days. The peas were kept in an incubator at 27° C. in total darkness except for the experimental exposures to light and for a momentary exposure to very weak blue light necessary when the water was changed; at the latter times illumination was from a two-cell flashlight fitted with a Klett-Summerson colorimeter filter (no. 50) transmitting at 470–530 mμ.

Pea stems were harvested under red light on the ninth day, using the third internode as recommended by VAN OVERBEEK and WENT (7). Stems in which the fourth internode was over 5 mm. long were not used. The split stems were washed in distilled water for 1 hour before being placed in the solutions of growth substances. Longer washing results in smaller curvatures of the stems in the solutions.

The solutions were made up with glass-distilled water, 2 ml. of 0.15 *M* phosphate buffer at pH 7.0 being included for each 100 ml. of solution. The response to growth substance was found to be greater at a pH value of 7.0 than at a pH value of 5.0. All experiments, therefore, were carried out at a pH value of 7.0. No beneficial effects were obtained from the addition of KCl or sucrose (4), so these were not included in the test solutions. Since the sodium salt of alpha-naphthaleneacetic acid ($C_{12}H_9O_2Na \cdot 4H_2O$—hereafter designated as SNA) is used as the standard of comparison in chemical screening of growth substances in this laboratory, all tests were run with this compound as the growth-regulating material. The results reported have been confirmed, however, for indoleacetic acid and other active compounds.

Five or ten replicate stems were used for each of the treatments. Shadowgraphs of the stems were taken after 16–24 hours in the test solutions in the dark. Inward curvature of the ten to twenty arms of the split stems was measured by the "point of inflection" method of WENT and THIMANN (12). Mean values are reported, together with the standard error of the mean (tables 1, 2).

Results and discussion

EFFECT OF LENGTH OF SINGLE EXPOSURE OF SEEDLINGS TO RED LIGHT.—It is evident from table 1 that red-light exposure on the day preceding harvest had a very marked effect on the response of split pea stems to SNA. Where little or no light reached the stems, curvature was very poor. At least 1 hour, and preferably as much as 4 hours, of red light (Mazda incandescent ruby-glass bulb transmitting above 600 mμ) of 10 foot-candles intensity on the day before harvest was needed to induce a maximum

response. All light-intensity measurements were made at the base of the seedlings, using a Weston Illumination Meter Model 603 calibrated with a tungsten lamp at 3000° K.

TABLE 1

RESPONSE OF SPLIT PEA STEMS TO SODIUM ALPHA-NAPHTHALENEACETATE (SNA) AS AFFECTED BY LENGTH OF EXPOSURE TO RED LIGHT* DURING GROWTH

Length of exposure (hr.)	Starting time of exposure (hr. before harvest)	No. of arms measured	Degrees curvature in different concentrations of SNA		
			10 p.p.m.	100 p.p.m.	200 p.p.m.
0	24	10	43 ± 6		48 ± 10
½			107 ± 37		129 ± 41
1			159 ± 27		322 ± 36
2			209 ± 21		234 ± 27
4			243 ± 34		348 ± 46
6			176 ± 56		201 ± 36
0	24	10		14 ± 5	
½				205 ± 30	
1				236 ± 40	
2				196 ± 34	
4				194 ± 46	
8				223 ± 33	
0	30	20		56 ± 9	
¼				259 ± 31	
½				278 ± 25	
1				335 ± 21	
1½				293 ± 21	
2				258 ± 27	
4				339 ± 28	
7½				385 ± 21	

* 10 foot-candles (60-watt Mazda incandescent ruby-glass bulb in reflector at 24 inches distance).

Seedlings exposed to red light and controls kept in the dark differed in the manner in which the split stems curved in response to growth substances. In the controls the curvature was confined to the tip end of the section, while in the light-exposed seedlings the stem response began immediately above the base of the cut (fig. 1).

EFFECT OF TIME LAPSE BETWEEN A SINGLE LIGHT EXPOSURE AND HARVEST.—The effect of prior exposure to light in enhancing curvature in the pea test was greatest when the exposure was started some 32 hours before harvest (table 2). A much shorter or longer time lapse between exposure and harvest resulted in a greatly reduced response of the stems to the growth substance. Evidently the third internode—the part of the stem used in the test—must have attained a length of 1–4 cm. and must directly receive the light exposure to get the benefits of illumination. This suggests that

FIG. 1.—Typical pea-test shadowgraphs showing effect of prior exposure to red light on stem curvature in 100-p.p.m. sodium alpha-naphthaleneacetate. *A*, no light treatment; curvature slight and generally confined to the tip. *B*, single 4-hr. exposure; marked curvature beginning at base of cut with arms of split stems generally overlapping. *C*, brief exposure daily; marked curvature beginning at base of cut but generally no overlapping of arms. (½ natural size.)

chemical transport from the second internode is not involved.

The physiological reactions in the seedlings which result in greater sensitivity to growth substances and for which light appears to be the "trigger" are not very rapid, since a 32-hour lag is necessary for maximum effect. The alternative explanation, that the reactions are immediate but that the internode is at a stage of maximal light-sensitivity 32 hours before reaching the necessary length for harvesting, is unlikely. The ability of these shorter internodes to respond to growth substances was determined using the straight growth test with pea-stem sections. Here also a lag

was evident. Stems were exposed to red light for 4 hours and harvested 32, 24, 8, and 4 hours after starting the light treatment, and 20-mm. sections were immersed in 50-p.p.m. naphthaleneacetic acid in pH 7.0 buffer. Elongation (measured after 16–24 hours) of 35%, 12%, 9%, and 10%, respectively, was obtained, as compared with 15–20% for stems receiving no illumination. Illuminated control sections immersed in water elongated 5–10%. GALSTON and HAND (1), working with 5-mm. pea-stem sections, found that 1–90 minutes of sunlight immediately prior to harvest reduced elongation 30–50% in a solution containing 1 p.p.m. indoleacetic acid at pH 6.1.

TABLE 2

RESPONSE OF SPLIT PEA STEMS TO SODIUM ALPHA-NAPHTHALENEACETATE AS AFFECTED BY TIME LAPSE BETWEEN START OF EXPOSURE TO RED LIGHT* AND HARVEST. TEN STEMS WERE USED FOR EACH CONDITION

STARTING TIME OF 4-HOUR EXPOSURE PERIOD BEFORE HARVEST	DEGREES CURVATURE IN 100-P.P.M. SOLUTION OF SNA (AVERAGE OF 20 ARMS)	
	Test no. 11	Test no. 15
0	18± 6	.....
4 hr.	17± 5	.....
8 hr.	85±13	22± 7
16 hr.	164±20	171±21
24 hr.	289±24	197±28
32 hr.	.....	314±28
40 hr.	.....	99±12
48 hr.	37± 7	85±14
3 days	23± 8	.....
4 days	14± 5	.....
5 days	14± 5	.....

* 10 foot-candles (60-watt Mazda incandescent ruby-glass bulb in reflector at 24 inches distance).

EFFECT OF MULTIPLE INTERMITTENT LIGHT EXPOSURES.—When daily exposures of red light ranging from ½ to 120 minutes were tried, the effect was marked leaf development and great elongation of the fourth internode at the usual harvest time (ninth day), so that this section was 3 cm. or more in length instead of the desired length of no more than a few millimeters. If the fourth rather than the third internode was harvested following such intermittent light treatments, however, it gave a satisfactory pea test. Moving the harvest up to the eighth day, when the fourth internode was still short and the third internode was 4.5–7.0 cm. in length, proved a satisfactory procedure. Daily ½-minute light exposures did not induce so much response as did 5-minute (or, still better, 20–40-minute) exposures.

The arms of these stems exposed to intermittent light remained at an appreciable angle to each other after immersion in solution of growth substance. Thus, in contrast to stems with a single exposure to light, the arms of these split stems did not overlap even when very marked inward curvature was obtained (fig. 1).

EFFECT OF LIGHT AT DIFFERENT WAVE LENGTHS.—Equal intensities of white light (tungsten lamp), orange light (Corning filter 348, transmitting above 550 mμ), and red light (Corning filter 242, transmitting above 610 mμ) induced similar responses in the pea test. Blue light (copper sulfate solution as the filter) proved to be much less effective under similar conditions of exposure. Ultraviolet radiation (mercury vapor lamp) also proved much less effective than red light. This similarity in spectral sensitivity for WENT's pea test and for leaf and stem growth of etiolated pea seedlings (3) suggests that these and other processes (such as photoperiodism) have a common control mechanism, which still remains to be elucidated.

In our hands, control of light exposure during growth of the seedlings has greatly increased the curvature in the pea test and has eliminated the occasional batches which do not respond to growth sub-

stances. It is believed that the pronounced effect of a preharvest exposure to red light on the response of the pea stems to growth substances may account for the wide discrepancies in the literature on the reported activities of various compounds as well as for the considerable variations in curvature from day to day or between laboratories. Since similar variations are experienced in the test for growth substances using *Avena* coleoptiles, the growth of which is known to be affected by red light (8) such as is used in the physiological darkroom, it might also be profitable to investigate the need for control of darkroom illumination in the *Avena* test.

Summary

1. Light has a marked effect on the response of the seedlings in the pea test for detecting growth-regulating activity of chemicals. Split stems from seedlings grown in complete darkness give little or no response to solutions of growth substances, while pre-illumination is effective in securing a response. The time of exposure and amount and quality of the light are all critical in obtaining maximal response.

2. Red light is most effective; single exposures should be started 32 hours prior to harvesting the pea stems. At an intensity of 10 foot-candles, a 4-hour exposure to red light was found to result in the greatest curvature in the pea test.

3. A preharvest exposure of the seedlings to red light greatly increases the curvature in the pea test and eliminates occasional batches which otherwise would not respond to growth substances. Although the reproducibility of the pea test still leaves something to be desired in assigning a numerical rating of activity to test compounds, the present study does indicate the importance of the factor of light in controlling response.

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PERSISTENCE OF 2,4-D TOXICITY IN HAWAIIAN SOILS[1]

ERNEST K. AKAMINE

Introduction

The widespread application of 2,4-dichlorophenoxyacetic acid (2,4-D) to soil for the control of weed emergence has created the important problem of its persistence in soils. Its toxicity has persisted for 10 days to 18 months (**5, 6, 11, 12, 13, 15, 22, 23, 24, 25, 27, 28, 31, 32, 35**). Many investigators have attempted to correlate the persistence of 2,4-D toxicity in soils with various factors, including certain physical and chemical properties, moisture content, temperature, organic matter content, microorganisms, and pH (**1, 2, 3, 4, 5, 7, 8, 9, 10, 14, 16, 17, 18, 19, 20, 21, 24, 26, 29, 30, 33, 34, 35, 36**).

In the present paper, data are presented from experiments designed to study the persistence of 2,4-D toxicity in various Hawaiian soils.

Material and methods

Soils from five locations (Kailua, Koko Head, Lualualei, Poamoho Experimental Farm of the University of Hawaii Agricultural Experiment Station, and University of Hawaii Farm) on the island of Oahu, Hawaiian Islands, were used. They represent typical agricultural soil types of the island and were obtained from areas which were continuously under vegetable cultivation. The Kailua soil, almost wholly a coarse, coral sand, is very open and porous, alkaline (70%–90% $CaCO_3$), and low in organic matter content. The Koko Head soil, an alluvial silty clay loam formed under rather limited rainfall, is open, friable, light reddish brown in color, normally slightly alkaline, and low in organic matter content. It is a youthful soil, consisting mostly of primary minerals. The Lualualei soil, a black alluvium with poor internal drainage, is a plastic silty clay which is sticky when wet and cracks upon drying but is easily dispersed by cultivation when dry. Its pH reaction is alkaline; its organic matter is well dispersed but low in amount. The soil from the Poamoho Experimental Farm of the University of Hawaii Agricultural Experiment Station (hereafter referred to as Poamoho soil) is a residual silty clay —a dark-red manganiferous kaolinitic clay with an acid reaction and low organic matter content. It does not exhibit the stickiness usually associated with clays, and its internal drainage is relatively good. The University of Hawaii Farm soil (hereafter referred to as University soil) is a dark-brown, loose, alluvial, silty clay loam. Because of the addition of chicken manure which contained hydrated lime, the organic matter content and pH (slightly alkaline) of the sample are higher than normal for the type.

In order to study the influence of climatic factors on the persistence of 2,4-D toxicity in these soils, indicator crops were planted at 2-week intervals in 2,4-D–treated soils in three series during the year. New lots of all five soils were used in each series except series I, from

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which the University soil was omitted. Series I was continued from November 12, 1947, to March 3, 1948; series II from March 10 to June 30, 1948; and series III from July 1 to October 21, 1948. The soils were placed in flats (12 × 17½ inches in area and 5¼ inches deep) in the greenhouse. Two indicator crops—Bountiful garden bush bean (*Phaseolus vulgaris* var. *humilis* Alef.) and Break O'Day tomato (*Lycopersicon esculentum* Mill.)—were then planted in drills. 2,4-D was applied as a dust to the soil surface at the rate of 10 pounds per acre. Three flats treated with 2,4-D and three untreated ones were prepared with each soil. For each flat one hundred tomato and fifty bean seeds were used. To eliminate the factor of leaching, water was applied in an amount just sufficient to allow for normal germination of the seeds and normal growth of the seedlings. At the end of 2 weeks the crops were harvested, and the number of normal and injured seedlings as determined by the emergence and appearance of the aerial organs of the seedlings was recorded. The next crop was planted in the same soils immediately after harvest. Thereafter, successive croppings were made until the emergence of normal seedlings in 2,4-D–treated soils was at least 95% that of the control. Soil temperatures (1 inch below soil surface) were recorded daily at 8:00 A.M. and at approximately the hottest time of the day. Records on weed emergence were kept for each cropping. The possibility of the herbicide having moved to the bottom of the soil mass was investigated at the conclusion of each series. Each flat was inverted, and the soil at the bottom of the flat was transferred to another flat. Indicator crops were then planted to observe any residual 2,4-D toxicity.

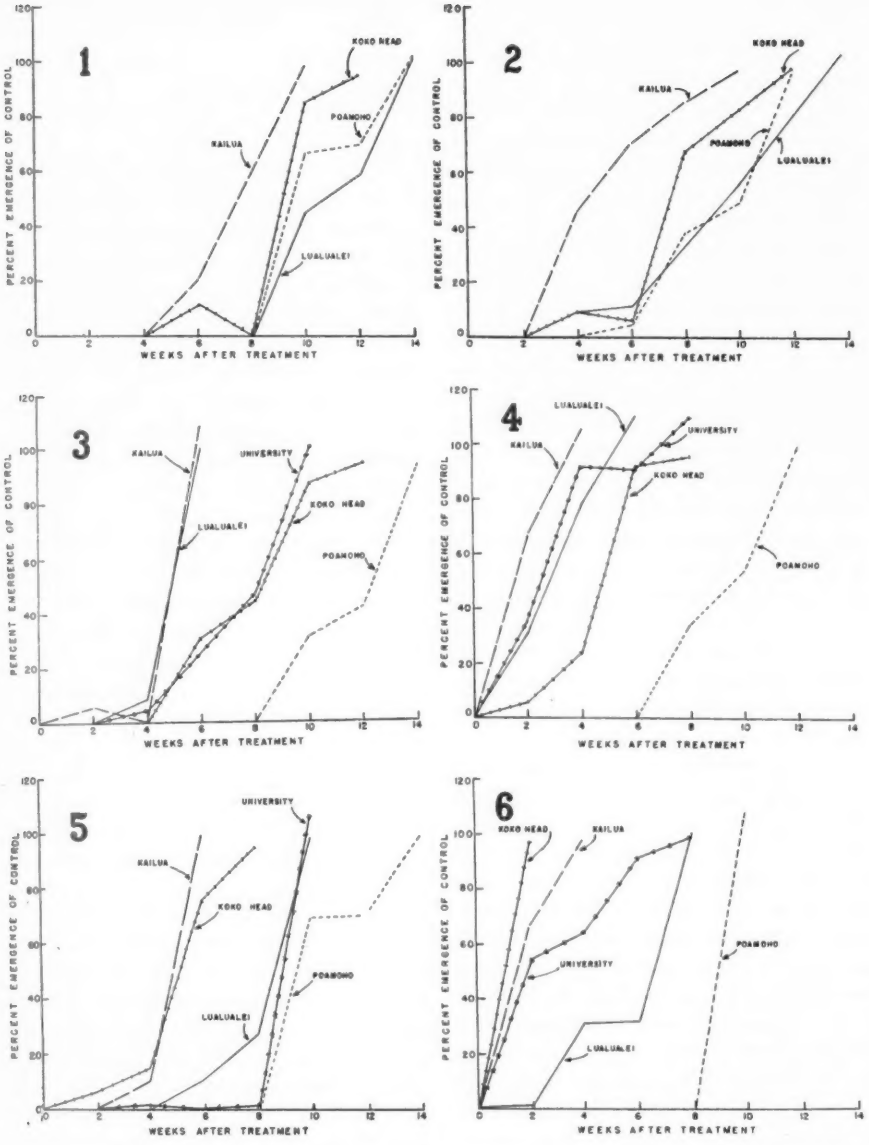
Attempts were made to correlate the persistence of 2,4-D toxicity with various soil factors: temperature, pH, fertility, organic matter content, microörganisms, and physical properties.

Results

Data on emergence

For all series only the results on emergence of normal seedlings of the indicator crops are presented (figs. 1–6). The trend of the data for emergence of injured seedlings was inverse to that for emergence of normal seedlings. Considerable variation among the different soils in their capacity to retain 2,4-D in active form is indicated by the data on emergence of normal bean and tomato seedlings in treated soils for all series. This variation among soils was similar in series I and II as tested by either crop. The data also indicate in general that bean is a more sensitive test plant than tomato, since soils lost their toxicity for tomato more rapidly. In general the results for series I and II were similar except for two points: (*a*) the position of the curve for Lualualei soil relative to the positions of the curves for the other soils and (*b*) in all soils except one (Poamoho) the rate of dissipation of 2,4-D toxicity in series II was more rapid than in series I.

In series III the bean and tomato indicator crops reacted somewhat differently (figs. 5, 6), but, if the soils are grouped on the basis of rapidity of dissipation of 2,4-D toxicity, the order of the groups is somewhat similar regardless of the indicator species employed. The position of the curves of the Lualualei soil relative to the positions of the curves of the other soils is different from either series I or II. When the curves of series III (figs. 5, 6) are superimposed on those of series II (figs. 3, 4) for each indicator species, it is seen that some pairs of



FIGS. 1–6.—Emergence of normal seedlings in 2,4 D–treated soils as percentage of emergence in untreated control soils. Figs. 1, 3, 5, bean seedlings; figs. 2, 4, 6, tomato seedlings, in series I, II, and III, respectively.

curves have different positions, while others have not. With tomato as the indicator species, most soils in series III generally lost their toxicity slightly more rapidly than the same soils in series II. With bean as the indicator species, there was less difference in the dissipation rate of the two series.

At the end of all series the test bean and tomato plants gave no indication of the presence of 2,4-D in the soils at the bottom of the flats. Weed emergence was controlled in all the 2,4-D–treated soils.

Factors of 2,4-D persistence

The differences in the persistence of 2,4-D toxicity among the three series seem to be related to differences in the mean daily soil temperatures (table 1); the higher the soil temperature, the more rapid was the rate of dissipation of 2,4-D (figs. 1–6). Differences in temperatures among the soils in any given series, however, are not considered to be of the magnitude to account significantly for their differences in persistence of 2,4-D toxicity.

Those soils which had high pH values as determined with the Beckman pH meter were the ones from which 2,4-D toxicity disappeared most rapidly (table 2; figs. 1–6).

The effect of an induced change in pH value of a soil (Poamoho) on the rate of dissipation of 2,4-D was studied. The pH value was 5.89 before treatment with cal-

TABLE 1

Mean daily soil temperatures (° C.) at depth of 1 inch

Soil	Series I		Series II		Series III	
	8:00 A.M.	Maximum	8:00 A.M.	Maximum	8:00 A.M.	Maximum
Lualualei	19.8	27.7	21.9	31.4	24.5	34.9
Koko Head	19.6	27.3	22.9	31.7	24.2	33.6
Poamoho	19.8	27.6	23.4	32.7	24.7	34.1
Kailua	19.7	27.3	21.8	30.2	23.7	32.8
University	.....	.....	23.0	31.8	24.5	34.2
Average	19.7	27.5	22.6	31.6	24.3	33.9

TABLE 2

Data on tested soils

Soil	pH			Organic matter content (%)			Relative aerobic bacterial count, series I (No. bacteria/gm. soil)	% adsorption* of 2,4-D, series III
	Series I	Series II	Series III	Series I	Series II	Series III		
Lualualei	7.90	8.46	7.82	2.46	2.49	2.32	602,500	0
Koko Head	6.26	7.50	7.50	3.86	4.37	3.30	1,560,000	0
Poamoho	5.75	5.88	5.89	3.13	3.35	2.82	865,000	7.54
Kailua	8.27	8.38	8.26	2.73	2.68	2.28	1,157,500	0
University	.....	7.94	7.31	.....	5.42	5.25	.....	36.23

* Spectrophotometric determinations.

cium hydroxide at the rate of 3000 pounds per 2,000,000 pounds air-dry soil. After liming, the soil was stored moist in the greenhouse for approximately a month before using. Its pH value was then 7.22. 2,4-D was then applied as a dust at the rate of 10 pounds per acre to the soil surface in test flats. The time required for 2,4-D toxicity to disappear as determined biologically with successive plantings of bean and tomato was the same for limed and unlimed soil.

In general, the organic matter content (Walkley's method) of the different soils used was relatively low and was similar for all the soils except the University soil, which contained chicken manure (table 2). The values cannot be correlated with the rate of disappearance of 2,4-D toxicity.

Relative aerobic bacterial counts (dilution method) made on samples of the soils in one series indicated variation in the number of these microörganisms in the different soils (table 2) and appear to be correlated with the rate of disappearance of 2,4-D toxicity.

Except for the organic matter determination, no other chemical analysis was made to measure fertility. In an experiment to determine the effect of fertilization on the persistence of 2,4-D toxicity, Poamoho soil was fertilized with a commercial fertilizer (4–12–8) at the rate of 1 ton per acre (worked into the soil) and then dusted with 2,4-D at the rate of 10 pounds per acre. Another lot was treated with 2,4-D, but no fertilizer was added. There was no significant difference in the rate of dissipation of the herbicide in the two lots as indicated by the emergence and appearance of bean and tomato seedlings.

Some of the physical properties of the soils employed in these studies were described previously. Properties varied considerably among the soils and undoubtedly affected the leachability and other factors. Since by careful application of water the 2,4-D was not allowed to leach out of the soils, leachability was not a factor determining persistence of 2,4-D toxicity. The ability of the soils to adsorb 2,4-D was determined spectrophotometrically (**3**), but the data obtained (table 2) do not seem to be correlated with 2,4-D persistence.

Discussion

Other investigators (**2, 5, 16, 24**) have shown that high soil temperatures are more conducive to dissipation of 2,4-D toxicity from soils than low temperatures. In the present work, comparisons among the three series of experiments—conducted during different seasons—indicated a similar relationship between temperature and the rate of 2,4-D dissipation for all soils.

The persistence of 2,4-D toxicity among the soils under study varied considerably (figs. 1–6). In general, the following order of soils, according to their capacity to inactivate the herbicide—although not absolute for each series or each test crop—seems valid: Kailua, Koko Head, Lualualei, University, and Poamoho.

Differences in temperatures among the soils in any one series were not considered of the magnitude to account for their differences in persistence of 2,4-D toxicity.

High pH values were associated with more rapid dissipation of 2,4-D. This does not appear to be in agreement with the findings of CRAFTS (**5**), who stated that 2,4-D breakdown is slower in neutral and alkaline soils than in acid ones. This is also not in agreement with the findings of KRIES (**17**), who reported that 2,4-D persisted for a longer period in limed than in unlimed soil. Furthermore,

WEAVER (**33, 34**) stated that perhaps acid soils have higher adsorptive capacities for 2,4-D than basic ones. An increase in pH of a normally acid soil (Poamoho) did not alter the persistence of 2,4-D toxicity. This probably is in agreement with observations made by HANKS (**14**), who found that leachates from limed and unlimed soils showed no significant difference in 2,4-D toxicity, and with observations made by JORGENSEN and HAMNER (**16**), who reported that differences in soil pH produced no effect on the rate of loss of toxicity of this compound.

By increasing the organic matter content by addition of leaf mold to limed soil, KRIES (**17**) was successful in reducing the persistence of 2,4-D. A light application of manure to soil low in organic matter hastened the inactivation of 2,4-D (**2**). KRONE and HAMNER (**18**) found that 2,4-D had a more lasting effect in mineral soils (sandy and sandy loam) than in muck soil. The organic matter content in all the Hawaiian soils studied is generally low and approximately the same; therefore, it apparently bears no relationship to the persistence of 2,4-D toxicity.

Although it is not known how 2,4-D affected the microörganisms in these soils, there seems to be some positive correlation between bacterial counts and the rate of 2,4-D dissipation. In reported studies, depending on the type of microörganisms, soil type, pH of medium, and other factors, soil microörganisms have been either affected adversely by 2,4-D (**4, 9, 10, 21, 26, 36**) or not affected at all (**19, 21, 29, 30, 36**).

It has been shown for one soil that increasing its fertility by addition of a commercial fertilizer did not influence its rate of inactivation of 2,4-D.

Although it has been stated that 2,4-D may be inactivated by adsorption or fixation by soil colloids (**8**) similarly to its inactivation by adsorption on carbon (**1, 20, 35**), the ability of the soils to adsorb 2,4-D, as determined spectrophotometrically, did not seem to be correlated with the retention of this herbicide in active form in the soils studied here.

Summary

1. The emergence and appearance of aerial organs of successive bean and tomato indicator crops, planted at 2-week intervals in series lasting over 11 months, were employed as criteria of persistence or disappearance of 2,4-dichlorophenoxyacetic acid in experiments on soils from five agricultural localities (Kailua, Koko Head, Lualualei, Poamoho Farm, and University Farm) on the island of Oahu, Hawaiian Islands.

2. Depending on soil type, environmental factors, and indicator crop used, 2,4-D toxicity was dissipated from the soils in 2–14 weeks. The 2,4-D had been applied as a surface dust at the rate of 10 pounds per acre.

3. The bean was more sensitive than the tomato as an indicator plant.

4. In general, the higher the soil temperature, the more rapid the rate of dissipation of 2,4-D. Differences in temperature among the soils during any given period, however, were not of an order to account for their differences in persistence of 2,4-D.

5. Those soils high in pH value inactivated 2,4-D more rapidly than those low in pH. A soil of pH 5.89, however, did not inactivate 2,4-D any more rapidly after liming to pH 7.22.

6. Aerobic bacterial count seemed to be related to 2,4-D persistence. Soils in which counts were high tended to retain the herbicide in active form for a shorter

period than those in which the counts were low.

7. Organic matter content, fertility (application of commercial fertilizer), and adsorptive capacity of the soils were not correlated with the degree of persistence of 2,4-D.

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ORIGIN AND NATURE OF HAUSTORIA IN LOBELIA CARDINALIS

K. SUBRAMANYAM

Introduction

In 1942 COOPER (**1**) published a paper on microsporogenesis and development of seed in *Lobelia cardinalis* L. He wrote: "The synergids and the antipodals function as micropylar and chalazal haustoria, respectively. The synergids become greatly enlarged and digest the cells of the integument about the micropyle. The nucellar cells at the chalazal end of the megagametophyte are digested by the antipodals, which later digest the cells of the adjoining nutritive tissue." After a critical study of the literature on the embryology of the Lobeliaceae, supplemented by his own observations on an Indian species of *Lobelia*, MAHESHWARI (**7, 8**) remarked that the occurrence of haustorial synergids and antipodal cells would be most unexpected in this family. He suggested that the haustorial structures are really formed from the terminal portions of the endosperm, a feature which is common in several families of the Sympetalae. Recently, ROSÉN (**11**) also criticized COOPER's observations on the haustorial nature of synergids and antipodals and stated: "It may be that COOPER has seen the micropylar and chalazal endosperm cells having the synergid-like shape and so he uncritically has taken them for synergids and antipodals." In the light of these comments it seemed desirable to make a fresh study of *L. cardinalis*.

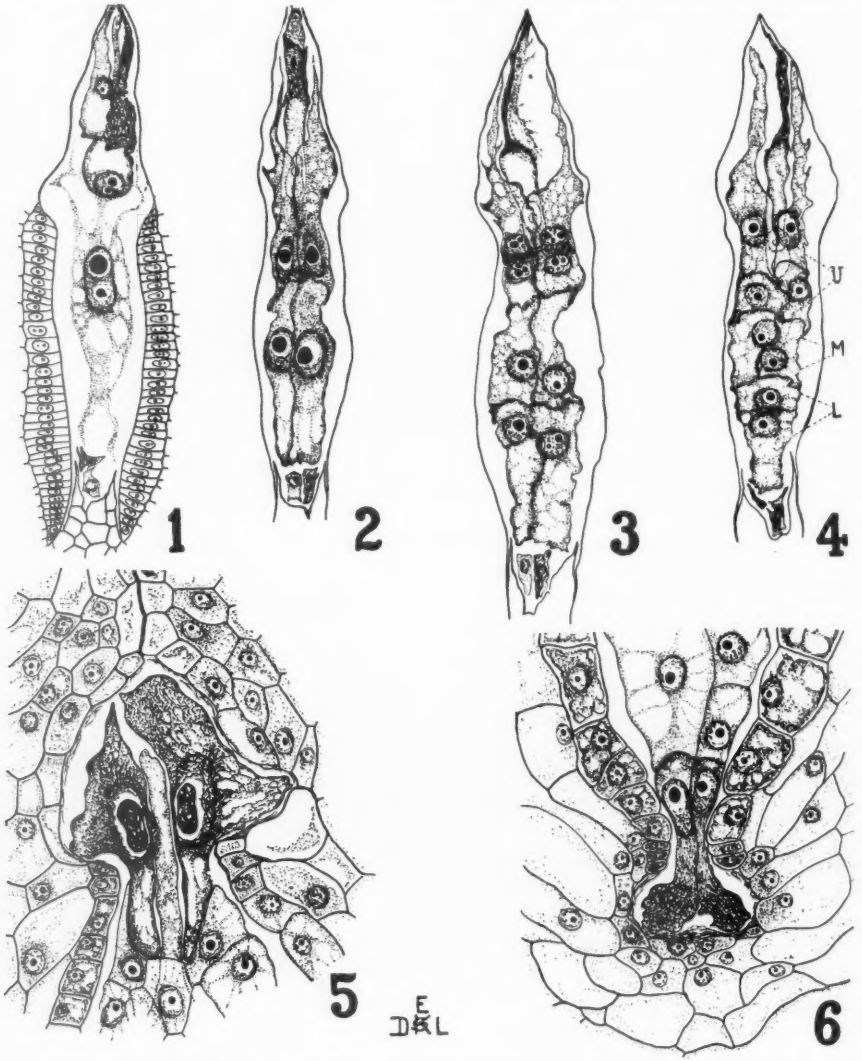
Material and methods

Material of open flowers and young fruits of this plant was collected at my request by Dr. V. PURI of Meerut (then

in the United States) from the campus of Cornell University on July 25, 1949, and fixed in formalin–acetic-alcohol. The sections were prepared and stained in the customary way.

Observations

The pollen tube enters the embryo sac through the micropyle and destroys one of the synergids (fig. 1) as in other members of this family (**2, 3, 4, 13**). Syngamy



FIGS. 1–6.—*Lobelia cardinalis*, stages in development of endosperm, ×970. Fig. 1, double fertilization. Fig. 2, four-celled endosperm. Fig. 3, eight-celled endosperm. Fig. 4, development of endosperm according to *Phyteuma*-type; *U*, upper chamber; *M*, middle chamber; *L*, lower chamber. Fig. 5, two-celled micropylar haustorium in advanced stage. Fig. 6, two-celled chalazal haustorium in advanced stage.

and triple fusion take place in the usual manner, after which the other synergid gradually shrivels and degenerates. MAHESHWARI (7) has pointed out that, although double fertilization is figured and said to take place normally, there is no trace of the pollen tube in any of COOPER's figures, either at the time of fertilization or after it.

The three antipodals are organized into definite cells. At the time of fertilization their nuclei show some tendency toward degeneration, but the cells persist during the early stages of endosperm development (figs. 2, 3, 4) as in other members of this family (5) and in the closely allied Campanulaceae (6) and Stylidiaceae (10, 11, 14, 15).

The primary endosperm nucleus divides much earlier than the zygote. The first wall is transverse, resulting in the formation of a micropylar and a chalazal chamber. Next, a vertical wall is laid down in each of these chambers, resulting in a four-celled endosperm (fig. 2). Transverse walls are then formed in each of the two tiers, first in the lower tier and then in the upper, as in *L. trialata* (4). The result is an eight-celled endosperm made up of four tiers of two cells each (fig. 3). The micropylar endosperm cells simulate the synergids in shape, and hence, as already pointed out by MAHESHWARI (7, 8) and ROSÉN (11), it is probable that COOPER mistook them for synergids.

At the eight-celled stage of the endosperm the two cells of the upper tier develop into the micropylar haustorium and the lower into the chalazal haustorium. The middle tiers by further divisions form the main body of the endosperm. Thus, the sequence of wall formation closely corresponds with that in the *Scutellaria*-type of SCHNARF (12). A similar course of divisions has been reported in other species of *Lobelia* (2, 4, 5, 8, 13) and also in members of the closely allied family Stylidiaceae (10, 11, 14, 15).

Figure 4 shows a deviation from the usual mode of endosperm development. Here there are five tiers of cells. The topmost tier consists of two cells and the lowest of only one. The two cells of the topmost tier form the micropylar haustorium, the single cell of the lowest tier differentiates into a chalazal haustorium, and the intervening tiers undergo further divisions to form the endosperm tissue. As to the origin of this condition, it appears probable that following the formation of a vertical wall in the primary micropylar chamber a transverse wall was laid down in the primary chalazal chamber, resulting in the formation of three tiers of cells, the upper (*U*) consisting of two cells and the middle (*M*) and the lower (*L*) of one cell each. Next, a transverse wall was laid down in the lower chamber, and, of the two cells thus formed, the basal functioned as the chalazal haustorium. The middle cell divided vertically, while the upper two cells divided transversely to form four cells, of which the topmost two cells became transformed into the two-celled micropylar haustorium. Such a sequence of wall formation closely corresponds with that in the *Phyteuma*-type (9) already described in certain members of the Lobeliaceae, such as *Laurentia tenella* (9), *Isotoma longiflora* (3), and *Lobelia pyramidalis* (13).

The micropylar haustorium (fig. 5) remains active for a long time. Its two cells become narrow toward the base and have a prominent lateral hump in the middle. Each cell has a large hypertrophied nucleus imbedded in finely vacuolate cytoplasm. This haustorium absorbs food from the micropylar cells of the integument.

The chalazal haustorium (fig. 6) also consists of two uninucleate cells. The distal ends of the two cells become extended and spread out laterally. This haustorium ceases its activities earlier than does the micropylar haustorium.

Between the two haustoria is the endosperm proper with conspicuous nuclei and dense contents.

Summary

1. In *Lobelia cardinalis* the pollen tube destroys one of the synergids. The other synergid degenerates after fertilization.

2. The antipodal cells persist during the early stages of endosperm development.

3. The endosperm is cellular and follows the *Scutellaria*-type of development as in other species of *Lobelia*. One case showing the *Phyteuma*-type of development was found.

4. At the eight-celled stage of the endosperm the two terminal cells at each end develop into the micropylar and the chalazal haustoria, respectively. The haustoria do not arise from the synergids and antipodals as described by COOPER.

It gives me great pleasure to thank Professor P. MAHESHWARI for valuable suggestions and for kindly reading the manuscript. I also thank Professor L. N. RAO for kind encouragement, Dr. V. PURI of Meerut for sending the material used in the present study, and the authorities of the National Institute of Sciences of India for the award of a research fellowship.

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TIME OF FLORAL DIFFERENTIATION OF THE OLIVE IN CALIFORNIA

H. T. HARTMANN

Introduction

A number of investigators have studied the time of floral differentiation in the olive (*Olea europaea* L.). In Italy MORETTINI (3) found the first evidence of floral primordia during the first half of March, preceding blooming by 2½ months. SAVASTANO and MARCUCCI (4), also in Italy, found that at the end of winter—10–15 days before flowering began—all the growing points of the "neuter" buds (described as neither a vegetative nor a fruit bud) started differentiation: the apical buds into leaf buds and the axillary buds into leaf or flower buds. KING (2) in California, in discussing the development of the floral axis of such fruits as the peach, plum, and apricot during the season preceding bloom, made the statement that "in the olive, however, corresponding stages of development are not apparent until about eight weeks before the flowers blossom." In Portugal, DE ALMEIDA (1) showed that flower-bud differentiation occurred in the Galega and Verdeal varieties during the early part of February, followed by blooming in May.

The present investigation was carried out to extend the work of KING (2). It was undertaken to determine whether differences existed in the time of floral initiation among the varieties grown in California, inasmuch as DE ALMEIDA (1) found this to be the case with certain varieties grown in Portugal. In addition, it was desirable to ascertain whether the time of floral initiation varied among the different olive-producing sections of the state.

Material and methods

Bud samples at the fourth or fifth node back from the terminal growing point were gathered during the 1946 season on dates approximately as follows: December 7, 1945; January 7, February 7 and 20, March 4, 12, 18, and 27, and April 3 and 10, 1946. About five nodes—with two buds per node—were taken at random around the tree at each sampling date and were placed immediately in vials containing a formalin–acetic acid–alcohol solution. Varieties used were Mission, Manzanillo, and Sevillano from Corning, Tehama County; Oroville, Butte County; Davis, Yolo County; Lindsay, Tulare County; and Riverside, Riverside County. Collections were made from the same trees throughout the season.

The buds were prepared for sectioning by treatment with the usual alcohol-xyol-paraffin series. Sections cut 10 μ thick were stained with safranin-fast green.

To determine whether the time of floral initiation varied appreciably from year to year, this work was repeated during the 1947 season. Bud samples were gathered on approximately the following dates: February 17, March 3, 10, 17, 24, and 31, April 7, 14, 21, and 28, and May 5 and 12. Again, the three varieties, Mission, Manzanillo, and Sevillano, were used, and collections were made, as before, from Corning, Oroville, Davis,

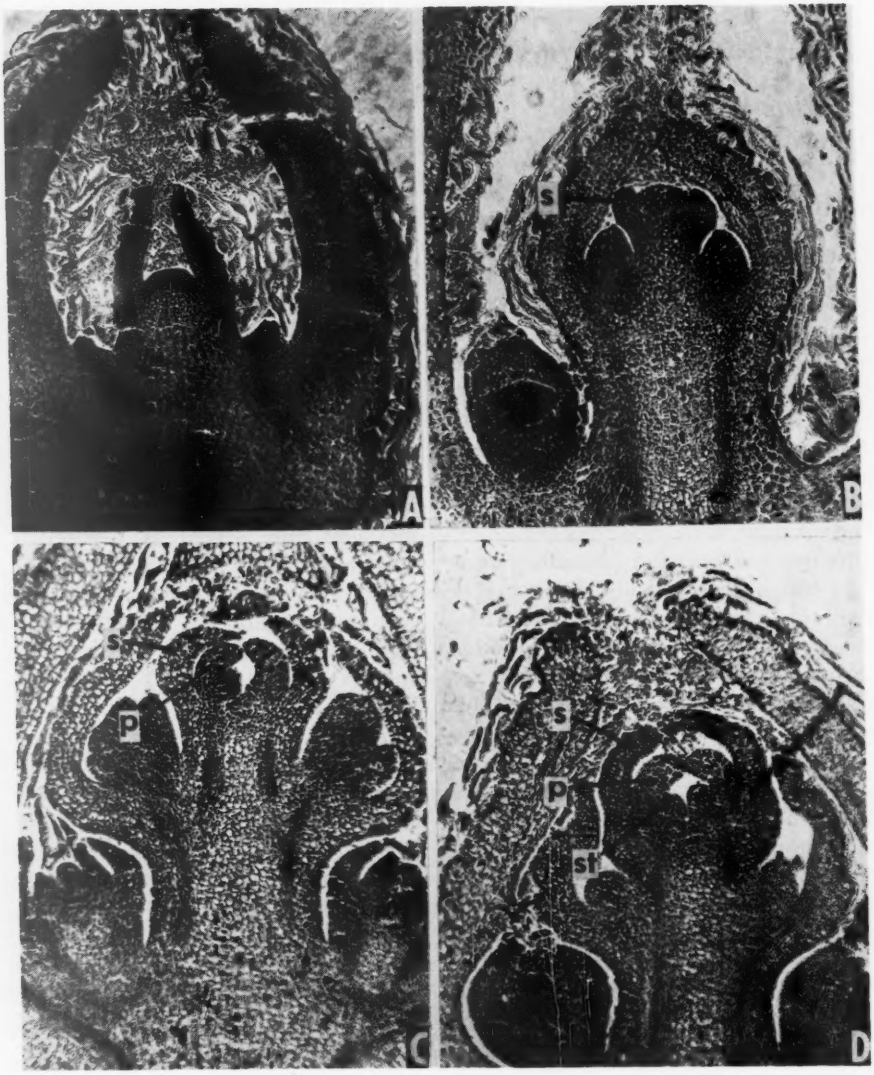

FIG. 1.—Development of floral parts in olive bud, ×65. *A*, vegetative bud; *B*, appearance of sepal primordia: *C*, sepal and petal primordia well developed; *D*, sepal and petal primordia in advanced state with appearance of stamen primordia; *s*, sepal; *p*, petal; *st*, stamen.

Lindsay, and Riverside. The buds were preserved, sectioned, and stained in 1947 in the same manner as in 1946. The complete series of collections, however, could not be obtained from the Corning district in 1947.

To determine whether there was an initiation of the inflorescence separate from the initiation of the floral parts, bud samples were collected from a Manzanillo tree at Davis at 2-week intervals from June 27 to December 5, 1947. Ten buds were taken at each sampling date and preserved in a formalin–acetic acid–alcohol solution. All the buds used were tagged on June 13 at the position of the third node back from the terminal growing point to insure that all buds in the series were initiated at approximately the same time.

In addition, another series was tagged on August 8, 1947, at the third node below the growing point, and samples were gathered at 2-week intervals until October 31, 1947. The buds in this series were, of course, approximately 2 months younger than those in the series previously mentioned.

TABLE 1

APPROXIMATE DATES OF FIRST EVIDENCE OF SEPAL PRIMORDIA AND OF FULL BLOOM FOR THREE OLIVE VARIETIES AT FIVE LOCALITIES IN CALIFORNIA

Locality	Variety					
	Mission		Manzanillo		Sevillano	
	Initiation of sepal primordia	Full bloom	Initiation of sepal primordia	Full bloom	Initiation of sepal primordia	Full bloom
	1946					
Corning	March 18	May 18	March 18	.....	March 18	May 16
Davis	March 27	May 21	March 18	May 23	March 18	May 25
Lindsay	March 12	May 10	March 27	May 17	March 27	May 22
Oroville	.....	May 17	March 27	May 14	March 18	May 11
Riverside	March 18	May 6	March 18	May 6	March 18	May 6
	1947					
Corning	March 13	.....	March 13	.....	March 13	.....
Davis	March 28	.....	Before April 10	.....	March 18	.....
Lindsay	March 4	.....	Before May 2	.....	March 4	.....
Oroville	.....	.....	Before May 2	.....	March 4	.....
Riverside	March 21	.....	March 5	.....	March 5	.....

Results

Floral differentiation was considered as beginning when longitudinal sections of the growing point of the bud showed a broadening of the tip into a more or less flattened conical mass, with the four sepal primordia arising as slight protuberances at the outer edges of the meristematic cone (fig. 1*B*).

The results for 1946 and 1947 are summarized in table 1, in which the collection dates are given when sepal pri-

mordia were first found. In some instances no definite results were obtained. For example, the slides examined from buds of the Mission variety at Oroville in 1946 as well as in 1947 gave no evidence of sepal primordia throughout the entire collection series. The tree used each year was apparently in the "off" year of an alternate-bearing cycle. No evidence of flower formation was seen in the Manzanillo variety at Davis in 1947 until April 10, when entire flowers were found in an advanced stage. Presumably all buds collected prior to this date happened to be in the vegetative state. This tree, it was noted later, had a very light bloom and set practically no fruit, likewise apparently being in the "off" year of an alternate-bearing cycle.

From the data obtained in 1946 and 1947 it is evident that the initiation of floral parts in the olive in California began, in general, sometime during the month of March, agreeing with the earlier statement of KING (2). A few sections were observed, however, in which floral parts were evident in February. There was no consistent difference in the time of differentiation among the three varieties studied or in the five localities of the state, even though the Corning and Riverside areas are about 550 miles apart. Using other varieties, however, DE ALMEIDA (1) reported differences up to one month in time of differentiation of some of the varieties studied in Portugal.

The dates of full bloom are given in table 1 for the trees from which bud samples were gathered in 1946. It is seen that floral initiation usually occurred approximately 2 months prior to full bloom.

The order of floral development of the olive is the same as described (5) for other fruit species. Following initiation of the sepals, petal primordia appeared, followed by the anthers, and finally the carpels. The last-named organs were first detected during the early part of April. Several stages in the development of the floral parts are shown in figure 1.

An examination of the series of buds collected for the inflorescence studies showed that the appearance of all the buds was the same, starting with the first collection on June 27. A bud in the vegetative state is shown in figure 1*A*. There was no change in the appearance of this type of bud until initiation of the floral parts began in March. The same observations were made for the series of buds tagged on June 13 and on August 8.

Summary

1. The beginning of floral differentiation in the olive in California was found in 1946 and 1947 to occur usually during the month of March, about 8 weeks before full bloom.

2. Little, if any, difference was noted in time of floral differentiation among the three varieties studied or among the five localities from which bud samples were collected.

3. No change occurred in the microscopic appearance of the bud from the time it was formed in June until the floral parts appeared the following March.

Appreciation is expressed to Drs. J. R. KING and R. M. BROOKS for their generous assistance in various parts of this study and to Mrs. THELDA BARTLEM, who prepared the slides.

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A NEWLY DEVISED COLCHICINE METHOD FOR INDUCING POLYPLOIDY IN RICE[1]

Artificial induction of polyploidy in rice has been attempted (1–8), but percentages of doubling were so low and uncertain that none of the previously used techniques can be used in rice-breeding. The coleoptile and first leaves envelop the shoot growing point in germinating seeds of monocotyledons so tightly that colchicine applied externally does not reach the area where cells are dividing. This paper describes an adequate technique, including proper concentrations of colchicine as well as technical manipulations, which provides for induction of polyploidy in rice quite as easily as in dicotyledons.

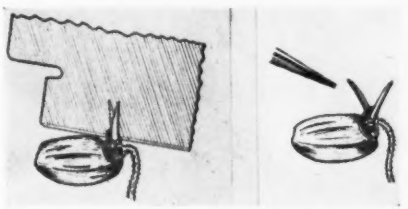

FIG. 1.—Technique for treating rice seedlings with colchicine. *Left*, shoot is split longitudinally from top to base. *Right*, cotton ball is inserted and colchicine solution applied.

Rough sorting of the $4x$ and $2x$ tissues in the first generation is advisable, and verification by chromosome counts, pollen mother cells, pollen grains, and stomatal sizes should be tried on the selected generations.

In this paper the author gives a description of the techniques and of the effects of colchicine on the young seedlings and some hints on the sorting-out of panicles with $4x$ tissues from the colchicine-treated individuals. The percentage of true tetraploids from grains produced on $4x$ tissues, and morphological and cytological observations, etc., on tetraploids raised from grains obtained from colchicine-treated plants, will be dealt with, in detail, in another paper.

METHOD.—The essential point of the method consists in exposing the shoot growing point of the germinating grain so that the colchicine can reach it easily and quickly. Hulled or unhulled grains are set to germinate in Petri dishes. When the shoot reaches a length of 3–5 mm., it is split longitudinally from top to base with a safety razor blade. Into the base of the split is inserted a small cotton ball or, preferably, blotting paper cut into squares about 0.5 mm. on a side. The cotton or paper is then wetted with colchicine solution (fig. 1).

TABLE 1

EFFECT OF COLCHICINE TREATMENT

Varieties	No. of seedlings treated	No. of seedlings surviving treatment	No. of individuals with $4x$ tissues
	0.1% colchicine solution		
11 Aikoku	10	2	2
1106 Konanto	10	6	4
1211 Philippines no. 3	10	5	4
1229 Russia no. 59	10	3	3
	0.05% colchicine solution		
126 Rikuha no. 132	10	8	4
152 Ginbozu	10	7	2
196 Asahi no. 1	10	9	1
961 Kairyo hagutai mochi	10	9	2
1091 Tanko hoira	10	7	1
Total	90	56	23

[1] Contributions from the Laboratory of Genetics, Biological Institute, Kyoto University, no. 187.

Treated young seedlings are transferred into a larger dish (25 × 5 cm.) containing a layer of cotton soaked with water and are covered over by blotting paper. The larger dish provides an optimum degree of humidity and also keeps the colchicine solution from evaporating too quickly.

FIG. 2.—Effect of colchicine on rice seedlings. *Left*, seedlings treated with 0.1% colchicine solution. *Right*, controls. (Photographed 2 weeks after treatment.)

A second application of colchicine is made the following day. Twenty-four hours later the cotton ball is removed from the split area, and the seedling is washed and carefully transplanted into the seedling bed.

EFFECTS OF COLCHICINE.—The most suitable concentration of colchicine for induction of polyploidy in rice seems to lie between 0.05 and 0.1% with two applications (table 1). An increase in colchicine concentration and/or in the number of applications was found to be detrimental and often fatal to the rice seedlings.

Success also depends largely upon the careful handling of the seedlings during the treatment, transplantation, and the first few weeks in the seedbed, for not only the colchicine but the cutting greatly weakens the plants.

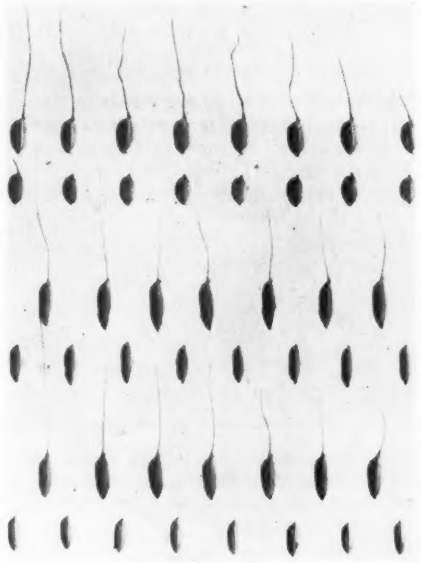

FIG. 3.—Comparison of diploid and tetraploid rice spikelets. Upper two rows: *above*, 4*x*; *below*, 2*x* Aikou. Center two rows: *above*, 4*x*; *below*, 2*x* Russia no. 59. Lower two rows: *above*, 4*x*; *below*, 2*x* Konanto.

After a few days the shoots swell, stop growing, and seem to remain in this state for some time; then they resume growth. During the early stages differences between treated seedlings and controls are marked in that the treated plants are shorter, darker green, thicker, and the leaves stouter (fig. 2). When tillering begins, however, these differences become less and less conspicuous, until at heading it is nearly impossible to distinguish between treated and control plants. In plants in which tillering is active the

treated individual seldom turns out to be completely tetraploid. Only some panicles and sometimes only a few grains in each panicle of each individual prove to be truly affected.

Panicles with $4x$ tissues often fail to emerge completely from the sheaths. Besides this striking character, the size and shape of ripe spikelets from $4x$ tissues are very helpful in distinguishing them from the diploid. Not only are they larger, with coarser hairs, but their awns, as a rule, are much longer than those of the original diploid (fig. 3).

Not all the spikelets which morphologically resemble tetraploids contain embryos which grow to be true tetraploid plants, because only the epidermis may be affected, and the internal parts may be composed of $2x$ tissues.

To Professor H. KIHARA's kind guidance, to Professor I. NISHIYAMA's continuous encouragement, and to Dr. I. HARAYOSHI's helpful suggestions, I wish to acknowledge my sincere thanks.
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MALEIC HYDRAZIDE AS A SPROUT INHIBITOR FOR POTATOES

Experiments begun at Cornell University in May, 1949, showed that maleic hydrazide injected directly into potato-tuber tissue was a very effective sprout inhibitor. Other tests showed that the same material applied externally had very little, if any, inhibiting ability.

On May 3, 1949, two lots of dormant Green Mountain tubers previously stored at 35° F. were selected. Each lot was then divided into three smaller lots, and the following treatments were applied: *1*, Check—ethyl alcohol only; *2*, 2500 p.p.m. diethanolamine salt of maleic hydrazide in ethyl alcohol; and *3*, 2500 p.p.m. methyl ester of naphthaleneacetic acid (MENA) in ethyl alcohol.

The three treatments were applied by two methods. The tubers were dipped into the solutions in the first case; in the second case ten toothpicks which had previously been soaked in the separate solutions were inserted half their length into a potato. Five replicates were used in all cases, and tubers were then stored at 50° F. under controlled conditions until data were taken on July 22, 1949. Results are shown in table 1 and figures 1 and 2.

TABLE 1

MALEIC HYDRAZIDE AS A POTATO SPROUT INHIBITOR

Treatment	Immersed in solution		Injected by toothpicks	
	No. of sprouts	Wt. of sprouts*	No. of sprouts	Wt. of sprouts*
1, Check	6.2	14.27	7.4	10.12
2, 2500 p.p.m. maleic hydrazide	6.0	13.80	0	0
3, 2500 p.p.m. MENA	0	0	1.0	1.17

* Weight of sprouts is given as percentage of original tuber weight. Data are averages per individual tuber.

The data indicate that maleic hydrazide could prove very effective as a sprout inhibitor once it was in the tuber tissue. Experiments are continuing on field applications designed to study the effect of such applications on plant and tuber responses.—ERNEST R. MARSHALL and ORA SMITH, *Department of Vegetable Crops, Cornell University, Ithaca, New York.*

FIGS. 1, 2.—Inhibition of potato sprouts by maleic hydrazide (MH) and methyl ester of naphthaleneacetic acid (MENA). Tubers treated on May 3, 1949, and photographed on July 22, 1949. Fig. 1 (*above*), treated by insertion of toothpicks soaked in (*1*) ethyl alcohol only; (*2*) 2500 p.p.m. MH; and (*3*) 2500 p.p.m. MENA. Fig. 2 (*below*), treated by immersion in same chemicals as in figure 1.